CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Biochemical Sciences

UNIVERSITY OF VIENNA

FACULTY CENTER FOR PHARMACY

Department of Pharmaceutical Chemistry

Laboratory of MacroMolecular Cancer Therapeutics

Reporter gene studies for nanoparticle mediated DNA and siRNA delivery

Diploma thesis

Charles University in Prague:

Supervisor: PharmDr. Anna Jirkovská, Ph.D.

University of Vienna:

Supervisor: Univ.Prof. Dipl. Ing. Dr. Manfred Ogris

Co-supervisor: Dr. Haider Sami, Ph.D.

Hradec Králové 2017 Kovářová Barbora

„I declare that this thesis is my original copyrighted work. All literature and other resources I used while processing are listed in the bibliography and properly cited. The thesis was not misused for obtaining the same or different academic degree."

„Prohlašuji, že tato práce je mým původním autorských dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci jsou řádně citovány. Práce nebyla použita k získání jiného nebo stejného titulu."

V Hradci Králové, dne Barbora Kovářová

Acknowledgement

I would like to express my thanks to Univ.Prof. Manfred Ogris for giving me the great opportunity to join his research team for elaborating this diploma thesis and for his advice.

Especially, I would like to express my gratitude to Dr. Haider Sami, Ph.D. for his guidance, advice and support during research and during writing this thesis. I really appreciate his encouraging and optimistic approach. I also want to thank the whole MMCT-team for friendly environment and all their help and advice.

I would like to express special thanks to PharmDr. Anna Jirkovská, Ph.D. for her understanding, help and useful advice not only during writing this thesis.

Also, I am grateful to my husband and parents for all their help, support and patience.

Finally, my thanks also belong to Erasmus plus project for financial support.

ABSTRAKT

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové, Katedra biochemických věd

University of Vienna, Faculty center for Pharmacy, Department of Pharmaceutical Chemistry, Laboratory of MacroMolecular Cancer Therapeutics

Kandidát: Barbora Kovářová

Školitel (Univerzita Karlova): PharmDr. Anna Jirkovská, Ph.D.

Školitel (University of Vienna): Univ. Prof. Dipl. Ing. Dr. Manfred Ogris

Konzultant (University of Vienna): Dr. Haider Sami, Ph.D.

Název diplomové práce: Studie nanočásticemi zprostředkovaného dopravování DNA a siRNA založené na reportérovém genu

Klíčová slova: transfekce, plasmidová DNA, siRNA, nanočástice

Genová terapie je nadějný obor, který nabízí potenciál k léčbě mnoha nevyléčitelných chorob. Je zprostředkována modulací genové exprese ve specifických buňkách pomocí dopravení exogenní nukleové kyseliny do těchto buněk. Jednou ze současných výzev dopravování nukleových kyselin je výzkum syntetických vektorů, které mají potenciál překonat nevýhody běžně používaných vektorů virových. Tato práce je zaměřena na různé typy nanočástic založených na polyethyleniminu pro dopravování plasmidové DNA (pDNA) a small interfering RNA (siRNA).

Spojení kontrastních zobrazovacích činidel se systémy pro dopravování genů nabízí mnoho výhod pro sledování procesu dopravování genů jak *in vivo* tak i *in vitro.* Kontrastní činidla založená na gadoliniu (např. kyselina gadoterová) vykazují potenciál pro aplikaci v zobrazování magnetickou rezonancí (MRI – magnetic resonance imaging). Nicméně spojení gadoterové kyseliny s polyethyleniminem může ovlivnit jeho schopnost transfekce. Za tímto účelem byly testovány polyplexy založené na lineárním polyethyleniminu (LPEI) značeném gadoterovou kyselinou (LPEI-DOTA-Gd) s cílem zhodnotit jejich účinnost v pDNA transfekci. Tato účinnost byla zkoumána na A549 a CT26 buňkách a vyhodnocena pomocí reportérového luciferázového testu (Firefly luciferase reporter gene assay) s výsledkem nevykazujícím negativní efekt konjugace s gadoterovou kyselinou na LPEI transfekční schopnost v porovnání s neznačeným LPEI.

V další části této práce byly testovány polyplexy pro dopravování siRNA založené na různých typech polyethyleniminu: lineárním (LPEI), rozvětveném (branched) (BPEI) a disulfidicky kroslinkovaném lineárním polyethyleniminu (c-LPEI), s cílem nalézt optimální parametry polyplexů a inkubační parametry pro účinné dopravování siRNA vedoucí k snížení exprese cíleného genu, v této práci genu pro světluščí (Firefly) luciferázu. Účinnost dopravování siRNA byla hodnocena na buňkách stabilně exprimujících gen pro světluščí luciferázu a vyhodnocena na základě poklesu aktivity této luciferázy. Polyplexy založené na BPEI byly nejúčinnější v dopravování siRNA vedoucímu ke snížení exprese cíleného genu, což nám umožnilo definovat optimální koncentraci, parametry polyplexů a inkubační parametry.

Posledním typem nanočástic testovaných pro dopravování siRNA byly tzv. "vrstvu po vrstvě sestavené zlaté nanočástice" složené z různých vrstev zahrnujících BPEI nebo c-LPEI. Výsledkem pilotního *in vitro* testování těchto nanočástic, také vyhodnoceného pomocí reportérového luciferázového testu, bylo specifikování rozsahu koncentrací z hlediska toxicity a potenciální účinnosti při snižování genové exprese, což může být využito v budoucích experimentech.

ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové, Department of Biochemical Sciences

University of Vienna, Faculty center for Pharmacy, Department of Pharmaceutical Chemistry, Laboratory of MacroMolecular Cancer Therapeutics

Candidate: Barbora Kovářová

Supervisor (Charles University): PharmDr. Anna Jirkovská, Ph.D.

Supervisor (University of Vienna): Univ. Prof. Dipl. Ing. Dr. Manfred Ogris

Co-supervisor (University of Vienna): Dr. Haider Sami, Ph.D.

Title of diploma thesis: Reporter gene studies for nanoparticle mediated DNA and siRNA delivery

Keywords: transfection, plasmid DNA, siRNA, nanoparticles

Gene therapy is a promising field offering potential in several currently incurable diseases. Gene therapy is mediated by modulation of gene expression in specific cells by delivering exogenous nucleic acids. One of current tasks of nucleic acid delivery is exploring several synthetic vectors which would have a potential to overcome the disadvantages of commonly used viral vectors. The present study focused on different types of polyethyleneimine-based nanoparticles for plasmid DNA (pDNA) and small interfering RNA (siRNA) delivery.

Integration of imaging contrast agents with gene delivery vehicles is advantageous for tracking the gene delivery process both *in vivo* and *in vitro*. Gadolinium based contrast agents (e.g. Gadoteric acid) have shown potential for magnetic resonance imaging (MRI) applications. However, conjugation of gadoteric acid to polyethyleneimine may affect its ability for transfection. Towards this goal, polyplexes based on linear polyethylenimine (LPEI) labelled with gadoteric acid (LPEI-DOTA-Gd) were tested for evaluation of pDNA transfection efficiency. The transfection efficiency, studied in A549 and CT26 cells and determined by Firefly luciferase reporter gene assay, showed that conjugation of Gadoteric acid did not cause any negative effect on LPEI transfection ability in comparison with unlabeled LPEI.

In another part of this work, polyplexes based on different types of polyethylenimine, i.e. linear (LPEI), branched (BPEI) and disulfide crosslinked (c-LPEI), were tested for

siRNA delivery with the aim to find optimal polyplex- and incubation parameters for efficient siRNA delivery, resulting in knockdown of the targeted gene (gene for Firefly luciferase in this case). The efficiency of siRNA delivery was investigated on cells stably expressing Firefly luciferase gene and estimated based on decrease in the luciferase activity as determined by Firefly luciferase reporter gene assay. BPEI-based polyplexes were the most efficient in siRNA delivery resulting in knockdown, enabling us also to define optimal concentration and some polyplex and incubation parameters.

Last type of nanoparticles tested for siRNA delivery were Layer-by-Layer assembled gold nanoparticles composed of different layers including BPEI or c-LPEI. Pilot *in vitro* testing of these nanoparticles, also evaluated by Firefly luciferase reporter gene assay, resulted in specifying of range of concentrations in terms of toxicity and potential knockdown efficiency for future experiments.

Table of contents

1. INTRODUCTION

1.1 Nucleic acid delivery

Gene therapy is a promising field, which offers a potential for the treatment of severe incurable diseases including cancer, cardiovascular diseases, acquired immune deficiency syndrome (AIDS), autoimmune and genetic disorders by modulating gene expression in specific cells. Such modulation is based on introducing exogenous nucleic acids into cells having a potential to modulate almost any sequence in the genome as well as any coding or non-coding RNA. Indirectly, gene transfer is used in the treatment of some diseases by its employment in production of proteins for therapeutic applications and vaccines. Furthermore, nucleic acid delivery is a crucial technique of *in vitro* studies of gene function and protein expression in molecular biology (Jin et al. 2014, Yin et al. 2014, Scholz and Wagner 2012).

Delivery of nucleic acids involved in gene therapy include delivery of DNA and mRNA for expressing therapeutic transgenes and delivery of several types of oligonucleotides, mostly more recently found, for blocking the expression of specific genes or correcting the activity of defective genes. Gene silencing can be achieved by delivery of antisense oligodeoxynucleotides (ODNs) or RNA interference (RNAi) nucleic acids including small interfering RNA (siRNA) and micro-RNA (miRNA) (Scholz and Wagner 2012, Lächelt and Wagner 2015). Conversely, endogenous mi-RNAs can be targeted by delivery of so-called antagomirs (Mattes et al. 2007). Delivery of splice switching oligonucleotides (SSOs) attracts attention for binding to genetically defective pre-mRNA and enabling expression of functional gene by changing the splicing pattern (Scholz and Wagner 2012). A protein-interacting nucleic acid represents another class which includes artificial nucleic acids called aptamers, binding target protein molecules with affinities compared to antibodies, and other nucleic acids interacting with immune response mostly mediated by their interaction with several receptors (Figure 1) (Lächelt and Wagner 2015).

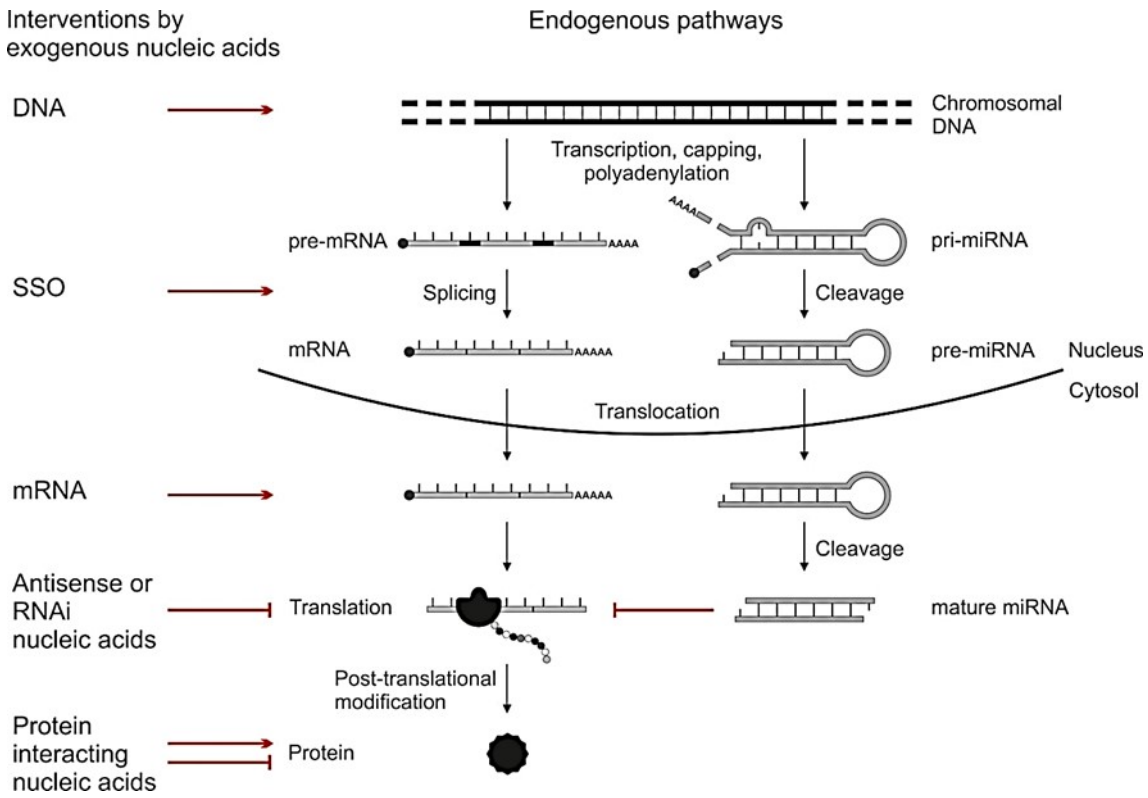

Figure 1. Stages of therapeutic intervention by nucleic acids.
DNA and mRNA are delivered for the expression of transgenes. SSO (splice-switching oligonucleotides) modulate splicing process. RNAi (RNA interference nucleic acids), such as miRNA (micro-RNA) and siRNA (small interfering RNA), and antisense oligodeoxynucleotides are introduced to cells for blocking the expression of targeted gene. Protein interacting nucleic acids can bind target proteins or interact with receptors and immune response.
Figure from Lächelt and Wagner 2015.

Nucleic acids are large macromolecules with negative charge. This prevents them from diffusing into cells directly through the plasma membrane (Jin et al. 2014, Yin et al. 2014). Moreover, they are prone to enzymatic degradation (Ibraheem 2014). To overcome these obstacles, various strategies for nucleic acids delivery have been developed. The two major strategies comprise either the use of viral vectors or non-viral approaches. Utilization of viruses carrying genes in their modified genome, e.g. modified retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAV) (Yin et al. 2014) has certain advantages, originating in natural viral life cycle, such as efficiency, specificity in entering cells (Jin et al. 2014) and long-term expression. Not surprisingly, viruses were the first carriers used to deliver therapeutic genes (Ibraheem et al. 2014) and still predominate in a number of gene therapy clinical trials carried out (Yin et al. 2014). Also, the first product for gene delivery, which reached the market authorization by the European Commission in 2012, alipogene tiparvovec (Glybera) uses an AAV vector

encoding a human lipoprotein lipase (LPL) gene variant (Lächelt and Wagner 2015). Unfortunately, several drawbacks are associated with viral vectors such as potential immunogenicity, carcinogenesis, limited DNA packaging and difficulty of large-scale production. Non-viral synthetic carriers, also called chemical vectors, have the potential to avoid many of these limitations, especially with respect to safety. Usually, these carriers, which in most cases are based on polymers, lipids (Yin et al. 2014), peptides (Scholz and Wagner 2012) or inorganic nanoparticles (Lächelt and Wagner 2015), also offer larger capacity for nucleic acids and easier production (Yin et al. 2014). Another non-viral approach for nucleic acid delivery is represented by physical methods that facilitate penetration of nucleic acid into cell. These methods include e.g. electroporation, ultrasound, needle injection, gene gun (Ibraheem et al. 2014) and hydrostatic pressure (Lächelt and Wagner 2015).

1.2 Polyethylenimine for nucleic acid delivery

The negatively charged phosphodiester backbone of nucleic acids enables the electrostatic interaction with cationic polymers to form complexes called polyplexes. Several cationic polymers have been investigated for nucleic acid delivery such as polyethylenimine (PEI), chitosan, polylysine, polypropylenimine (PPI) and polyamidoamine-dendrimers (PAMAM) (Scholz and Wagner 2012, Jin et al. 2014, Ibraheem et al. 2014).

In this thesis, polyethylenimine was used as transfection agent (Figure 2). Two structural types of PEI are distinguished: linear (LPEI) and branched (BPEI) polyethylenimine. Both types have been investigated for pDNA delivery for which LPEI/pDNA complexes show better transfection efficacy than those with BPEI (Wightman et al. 2001). In contrast, BPEI can be more suitable for siRNA delivery than LPEI (Kwok and Hart 2011). This difference can be explained by the stability of complexes correlating with different size of pDNA, with a size of several kilo bp, and siRNA, having size about 21 to 23 bp and consequently offering less electrostatic interactions with PEI. As the branched PEI provide flexible structure and folding options it also shows superior siRNA complexation properties compared to LPEI. Similarly, BPEI/pDNA polyplexes has higher stability compared to already quite stable LPEI/pDNA, however, in this case the too high stability makes the BPEI/pDNA complexes inefficient carriers (Kwok and Hart 2011, Scholz and Wagner 2012).

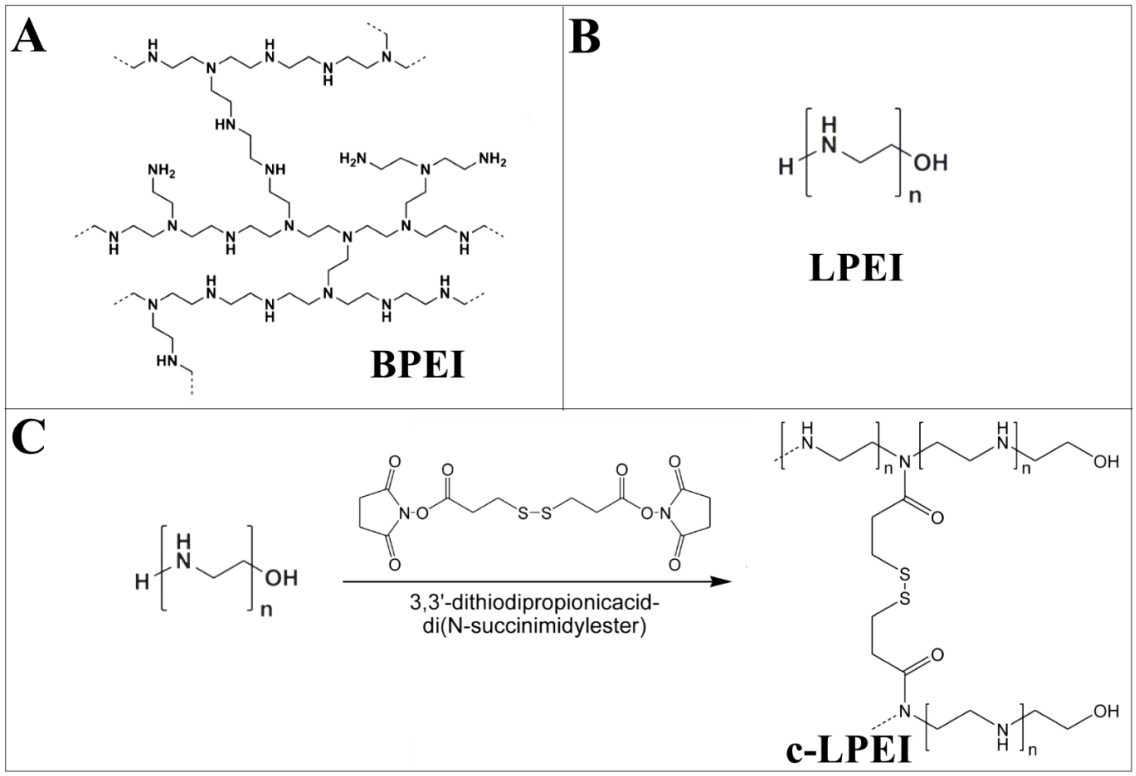

Figure 2. Types of polyethylenimine.
Types of polyethylenimine used in this thesis. BPEI = branched polyethylenimine, LPEI = linearpolyethylenimine, c-LPEI = disulfide crosslinked LPEI.
Figure adapted from Lächelt and Wagner (2015), Breuning et al. (2007).

Not only structural type but also molecular weight (MW) correlates with transfection efficacy which increases with the molecular weight of PEI (Breunig et al. 2007). Unfortunately, with higher molecular weight, toxicity of PEI also increases (Peng et al. 2008). Not only PEI, but also other polycations (e.g. PAMAM, polylysine) suffer from molecular weight dependent toxicity so their effective employment in transfection seemed to be contingent on finding a compromise between toxicity and efficacy. To overcome these limitations, crosslinking of well-tolerated low molecular (LMW) polymers by potentially biodegradable linkages into larger polymers has been introduced. Such linkages for PEI can be e.g. ester bonds, disulfides, ketals, imines and amide linkages. In general, these modifications achieved great reduction of toxicity with no impairment of efficacy or even with enhanced efficacy (Lächelt and Wagner 2015).

The disulfide cross-linking of LMW PEIs is especially attractive for its degradation in reductive intracellular environment. In this disulfide reduction, glutathione (GSH/GSSG) redox system plays a crucial role. Glutathione has also greater effect on redox potential than other redox couples such as nicotinamide adenine dinucleotide

phosphate ($NADPH/NADP^+$) or thioredoxin (TRX_{red}/TRX_{ox}). Intracellular concentration of reduced glutathione (GSH) is up to 1000-fold higher than the extracellular. Therefore, the nucleic acid delivery complex is stable outside cells while high GSH concentration enables rapid release of nucleic acids in the intracellular environment (Figure 3) (Peng et al. 2008, Son et al. 2012). As described by Breuning et al. (2007), this reduction is rapid and takes place not only in endolysosomes but also in cytoplasm compared to e.g. esters linkages with long hydrolysis half-lives and dependence on acid catalysis.

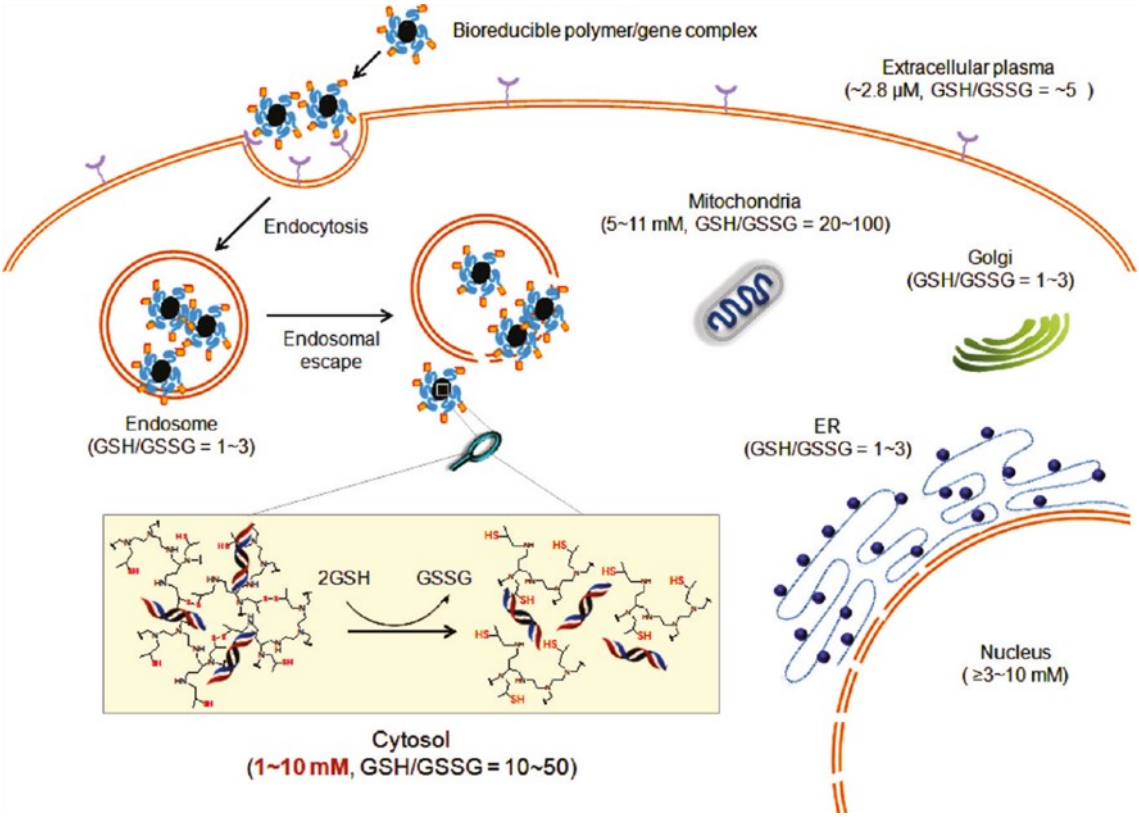

Figure 3. Scheme of intracelular reduction of bioreducible polymer/gene complex.
GSH/GSSG = reduced/oxidized form of glutathion.
Figure from Son et al. (2011).

Several approaches to crosslink PEIs by disulfide bonds have been applied either using cross-linkers with disulfide moieties or installing first thiol group to PEI followed by its oxidation. (Son et al. 2011). Disulfide crosslinked LPEI (c-LPEI) used in this thesis was synthetized from LMW LPEI using 3,3'-dithiopropionic acid di-(*N*-succinimidylester) (Lomant's reagent) as the linker (Figure 2), similarly as described by Breunig et al. (2007).

1.3 Characteristics of polyethylenimine-based polyplexes

The electrostatic interaction between nucleic acid and PEI results in formation of polyplexes with characteristics important for delivery pathways. Size of these nanoparticles is an important characteristic influencing mechanisms of uptake by cells, organs, passive tumor targeting and their renal excretion. For delivery of pDNA, formation of polyplexes enables the collapse of quite large (up to μm) pDNA molecule into smaller nanoparticles (30 - 100 nm) capable to enter cells by endocytic pathways. In case of siRNA, being already small molecule (2 - 7 nm), the goal of complex formation is not primary a reduction of the size but protection against nucleases and influencing of cellular uptake (Scholz and Wagner 2012). However, the size of created siRNA polyplexes can also differ from around 25 nm (Meyer et al. 2008) to several hundreds of nanometers (Scholz and Wagner 2012).

As observed for PEI/pDNA polyplexes, the size is influenced by polyplex characteristic called N/P ratio: the molar ratio of nitrogens in PEI and phosphates in nucleic acid. Polyplexes with low N/P ratios were almost neutral in charge with high aggregation tendency leading to larger particle size. Oppositely, higher N/P ratios led to formation of smaller polyplexes. In fact, the size dependence on N/P ratio was more pronounced for BPEI/pDNA polyplexes than for those using LPEI. The aggregation tendency is also influenced by other parameters such as DNA concentration and ionic strength of the buffer used for polyplex mixing.

At higher N/P ratios, also the presence of free PEI not complexed with nucleic acids must be considered influencing both transfection efficacy and toxicity (Scholz and Wagner 2012).

1.4 Delivery pathway of polymer-based nanoparticles for nucleic acid delivery

Cell uptake mechanism is determined by nanoparticles properties, especially by their size. Endocytic uptake is the preferred way for nanoparticles between 50 and 100 nm in size. Further, surface charge and attachment of ligands plays a role. Positively charged particles show electrostatic binding to negatively charged cell membranes. If the nanoparticle bears a targeting ligand, it can be also taken up into cell by receptor-mediated endocytosis (Scholz and Wagner 2012). As Kichler et al. (2001) explains, endocytosis of polyplexes is believed to be induced and accomplished soon after cellular association, that is within 4 hours.

Once taken up, carriers must escape from the endosomal pathway to release the nucleic acid. As endosomes mature, the action of proton pump acidifies their content (to pH 5-6) and in the end, they fuse with lysosomes (pH 4-5) resulting in degradation of their content. (Nguyen and Szoka 2012). However, requirements on the release differs depending on the place of action of the therapeutic nucleic acids. For example, siRNA and ODNs activity takes place in cytosol, whereas DNA needs to get to the nucleus (Scholz and Wagner 2012). The endosomal pathway in cells is heading from the periphery towards nucleus using a network of microtubules and motor proteins (Nguyen and Szoka 2012). Therefore, in contrast to siRNA delivering particles for which the early release from endosome is desired (Scholz and Wagner 2012), DNA delivering carriers should escape from endosome optimally in perinuclear region (Nguyen and Szoka 2012).

Cationic polymers have strong buffering capacity leading to the so called proton-sponge effect enabling the endosomal escape. While the proton pump acts to acidify the endosomal content, theses polymers capture protons like a sponge. This leads to further influx of protons followed by influx of Cl^- ions (Nguyen and Szoka 2012). Consequently, the following water influx causes osmotic pressure inside the endosomes resulting in bursting of lysosomes and release of their content (Taranejoo et al. 2015).

Apart from well-known proton-sponge effect, Nguyen and Szoka (2012) explain the umbrella hypothesis. The hypothesis describes conformation change of polymers with tertiary amine groups after their protonation. Whereas in complex with nucleic acid, held by electrostatic interactions, they are curled up in small particle, in excess of protons

in endosomal environment they spread out due to protonation of amine groups and their subsequent electrostatic repulsion.

Taking both mechanisms into consideration, for endosomal escape the main two characteristics of polymer are crucial: titrable amine groups at pH 5-7 and highly flexible structure (Nguyen and Szoka 2012).

1.5 RNA interference

RNA interference, first discovered in plants in 1980s, then in nematode *Ceanorhabditis elegans* and later in 2001 in mammalian cells, is a mechanism in which double-stranded RNA molecules, members of a family of non-coding RNAs, silence the expression of target genes on the post-transcriptional level. Major mediators of the interference are siRNA and miRNA (Ozcan et al. 2015).

Whereas miRNA originates endogenously from transcription of the genome, siRNAs are synthetic molecules. Both are small, double-stranded RNA molecules with miRNA usually about 22 base pairs (bp) long and siRNA, similarly, having 21 to 23 bp in length (Cai et al. 2009, Ozcan et al. 2015). A key role in the silencing mechanism plays the complex of Argonaut 2 and RNA-induced silencing complex (AGO2-RISC complex) that recognizes miRNA or siRNA, degrades one of their strands and use the other strand for guidance to the target mRNA to either bind and block it, in case of miRNA, or to cleave and degrade the mRNA, which is caused by siRNA. Another difference lies in complementarity to the target mRNA. Unlike the imperfect complementarity of miRNA, siRNA has perfectly or nearly perfectly complementary sequence to the target.

siRNA, used in this thesis, became great tool for studying gene function both *in vivo* and *in vitro* and attracts attention as new therapeutics (Ozcan et al. 2015). The number of siRNA therapeutics in clinical trials is steadily increasing (Lächelt and Wagner 2015). Examples of diseases treatments evaluated in the ongoing trials are solid tumors, advanced cancers like pancreatic ductal carcinoma, age-related macular degeneration (AMD), diabetic macular edema (DME), hepatitis B infection and others. One of the most advanced in clinical trials is Patisiran, a lipid formulation of siRNA targeted against transthyretin (TTR), for treatment of TTR mediated amyloidosis (Lorenzer et al. 2015). The phase 3 of clinical trials is ongoing with data anticipated by September 2017 (Patrick 2017).

Despite the potential of siRNA applications, many challenges remain, including off-targeted effects, poor cellular uptake and rapid degradation (Ozcan et al. 2015). Some initial clinical trials with local application of naked siRNA were even terminated, e.g. Bevasiranib clinical trial, the first trial with siRNA, was terminated for the poor efficacy (Lorenzer et al. 2015, Ozcan et al. 2015). Approaches to overcome these obstacles include rational design of siRNA and *in silico* selection, chemical modifications of siRNA and development of siRNA nanocarriers (Ozcan et al. 2015). Though chemical modifications of the siRNA backbone can result in preventing the rapid degradation, and some of them can also increase the cellular uptake, it has been found that many considerable changes interfere with activation of the intracellular RNAi machinery (Lorenzer et al. 2015). Development of nanocarriers have the potential to overcome not only the poor cellular uptake, but also the other challenges as well as they offer improvement in pharmacokinetics or reduction of siRNA-related toxicities. The most extensively studied are polymer- and lipid-based nanoparticles. Many of the siRNA therapeutics tested in clinical trials are formulated in lipid nanoparticle such as SNALP (stable nucleic acid lipid particle), AtuPLEX (a cationic lipoplex), vitamin A-coupled lipid nanoparticle and others. Studied polymeric nanocarriers include nanoparticles made of natural polymers such as chitosan and atelocollagen, that are characterized by their safety and have shown great efficiency for *in vivo* siRNA delivery, and nanoparticles based on synthetic polymer polyethylenimine. Polyethylenimine (PEI) offers advantages such as high efficiency and endosomal escape, however its use is limited by cytotoxic effects (Ozcan et al. 2015). Thus, biodegradable cross-linking of PEI, described in section 1.2, can help to get rid of the correlation between high efficiency and high toxicity.

Other nanoparticles such as quantum dots, carbon nanotubes, gold- and magnetic nanoparticles were also studied for siRNA delivery *in vitro* and *in vivo* to appraise the location of the nanoparticle and siRNA often with promising results (Ozcan et al. 2015, Kesharwani et al. 2012).

Another class of siRNA delivery approaches represents targeted delivery which is based on attaching ligands to the exterior surface of nanoparticle. Such ligands are e.g. functional peptides, aptamers or small organic molecules. Targeting has a great potential especially for tumor delivery using ligands such as transferrin receptor, prostate specific membrane antigen and folate receptor alpha (Ozcan et al. 2015).

1.6 Bioluminescence

Bioluminescence, a visible light emission by living organisms, is a biological phenomenon occurring among certain living organisms in aquatic as well as in terrestrial environment. Its biological function includes for example hunting prey, distracting predators or signaling e.g. for courtship. Light emission is a result of the decay of molecules in electronically excited state to the ground state. (Badr and Tannous 2011, Greer and Szalay 2001, Marques and Esteves da Silva 2009). Unlike fluorescence, luminescence does not require absorption of light, but a chemical reaction to gain the excited state of the light emitting substance (Fan and Wood 2007).

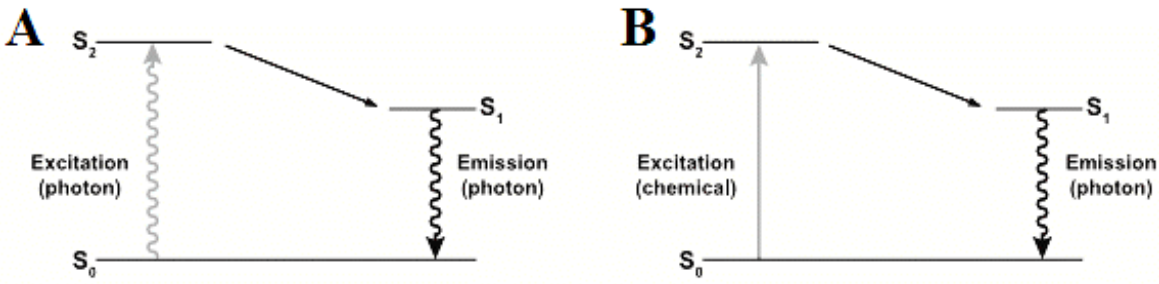

Figure 4. Comparison of fluorescence (A) and luminescence (B).
S_0 = ground state, S_1 = excited state after vibrational relaxation, S_2 = excited state.
Figure from Fan and Wood (2007).

The reaction is catalyzed by enzyme called luciferase and further requires a substrate generally called luciferin and oxygen. Some luciferases may require for their activity other cofactors, such as ATP or Mg^{2+}. Up to present, various luciferin-luciferases systems were defined ranging from bacterial *lux* genes (e.g. *Photorhabdus luminescens, Vibrio harveyi*) to eukaryotic luciferases from organisms such as algae, crustacean, annelids, cnidaria or beetles (Badr and Tannous 2011, Greer and Szalay 2002). In biomedical research three luciferases are most widely used: Renilla luciferase from sea pansy *Renilla reniformis*, Gaussia luciferase from marine copepod *Gaussia princeps* and Firefly (*Photinus pyralis*) luciferase (Badr and Tannous 2011). Gaussia and Renilla luciferases catalyse reaction of substrate coelenterazine resulting in the product coelenteramide and emission of blue light (with peak at 480 nm). Compared to Firefly luciferase reaction described below with substrate D-luciferin generating green light (562 nm) and producing oxyluciferin. Unlike the two others, Gaussia luciferase is a naturally secreted protein

and belongs to the smallest luciferases with a molecular weight of 19,9 kDa (Kaskova 2016, Badr and Tannous 2011).

Firefly luciferase is a 62 kDa enzyme (Conti et al. 1996) and catalyzes the reaction of its substrate D-luciferin, [(*S*)-2-(6′-hydroxy-2′-benzothiazolyl)-2-thiazoline-4-carboxylic acid], resulting in excited-state product that emits yellow-green light during its decay to the ground state. The reaction requires ATP, molecular oxygen and metallic cation and is described as two-step process. In first step, the carboxylate group of D-luciferin reacts with ATP-Mg^{2+} generating enzyme-bound intermediate luciferyl-adenylate while the pyrophosphate group (PPi, more precisely PPi-Mg^{2+}) is displaced. In the second step, oxidation and decarboxylation follows. The intermediate reacts with molecular oxygen resulting in displacing AMP group and formation of energy rich intermediate, the luciferin dioxetanone, that spontaneously break up generating CO_2 and oxyluciferin in excited state which is emitting photon while returning to the ground state (Marques and Esteves da Silva 2009, Conti et al. 1996).

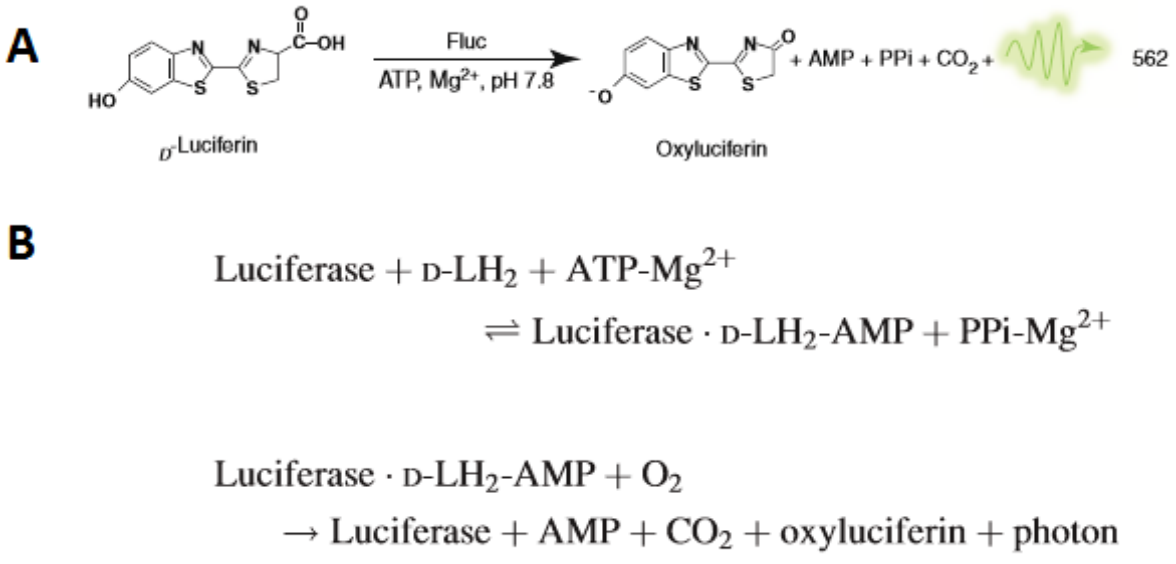

Figure 5. Bioluminescence reaction of Firefly luciferase.
Firefly luciferase catalyses reaction of substrate D-luciferin resulting in excited-state product oxyluciferin that emits yellow-green light (562 nm) during its decay to the ground state. The reaction requires other essential factors such as ATP and Mg^{2+}.
A: Scheme of the reaction. Figure adapted from Badr and Tannous (2011).
B: The reaction shown as two-step process. Figure from Marques and Esteves da Silva (2009).
Fluc = Firefly luciferase, PPi = pyrophosphate group, D-LH_2 = D-luciferin, Luciferase • D-LH_2-AMP = enzyme-bound intermediate luciferyl-adenylate.

Luciferases are commonly used as reporter genes, or, to be exact, are products of these genes. Reporter genes are genes whose expression can be easily measured and therefore are extensively applied in research for studying gene expression or as markers for studying transfection efficiency (Thermo Fisher Scientific 2017b). Such reporter gene assays are based on measuring changes in luciferase levels (Fan and Wood 2007). Thanks to its sensitivity, alongside the reporter gene assays, bioluminescent reactions are broadly used for applications including e.g. cell viability assays based on determining not the luciferase levels but the levels of ATP (Thermo Fisher Scientific 2017a). Another approach is to determine luciferin levels which is useful in measuring activities of an enzyme, e.g. cytochrome P450. In such assay, a pro-luciferin is applied which is converted by the measured enzyme into luciferin. Thus, the luminescent signal is dependent on activity of the measured enzyme (Fan and Wood 2007). Of great importance is also the employment of bioluminescence in *in vivo* imaging. This non-invasive imaging method uses labelling of molecules, pathogens, cells or disease models by luciferase and their visualization by addition of luciferin (Thermo Fisher Scientific 2017a).

2. AIMS OF THIS WORK

The present work deals with *in vitro* studies of nanoparticles mediated delivery of nucleic acids, namely plasmid DNA (pDNA) and small interfering RNA (siRNA), evaluated by Firefly luciferase based assay. Different types of nanoparticles were examined for nucleic acid delivery.

Polyplexes based on linear polyethylenimine (LPEI) labelled with gadoteric acid (DOTA-Gd; used as MRI contrast agents) were tested for evaluation of effect of gadoteric acid on pDNA transfection efficiency. Delivery of pDNA was mediated by polyplexes composed of either linear polyethylenimine (LPEI) or LPEI labelled with gadoteric acid (LPEI-DOTA-Gd), and pDNA encoding Firefly luciferase gene (using the plasmid pCpG-hCMV-EF1α-LucSH). The goal was to compare transfection ability of the LPEI-DOTA-Gd (which was newly synthetized for *in vivo* imaging in mice) with that of LPEI (as reported in Taschauer et al. (2016)).

Delivery of siRNA against Firefly luciferase gene (LucsiRNA) was mediated either by polyethylenimine-based polyplexes or by Layer-by-Layer assembled gold nanoparticles.

The aim of polyplex mediated siRNA delivery studies was to optimize parameters for the efficient delivery resulting in knockdown. These parameters include incubation parameters and polyplex parameters including usage of different types of polyethylenimine. The goal was also to test and compare LPEI, BPEI and also disulfide crosslinked LPEI (c-LPEI), the latter with promising biodegradable properties, for polyplex mediated siRNA delivery.

The aim of siRNA delivery mediated by Layer-by-Layer assembled gold nanoparticles was to perform pilot *in vitro* studies with the newly synthetized nanoparticles with comparison of the different nanoparticles composition containing BPEI or c-LPEI.

Several knockdown studies are frequently presented in current literature, however with different methods of presenting the data. Therefore, another goal of present work is to compare three routinely used methods of presenting the knockdown with highlighting their advantages and disadvantages.

Throughout the present work, the Firefly luciferase based reported gene assay is crucial to determine the efficiency of the delivery. Therefore, preparing and testing of buffer for the Luciferase assay was part of this work.

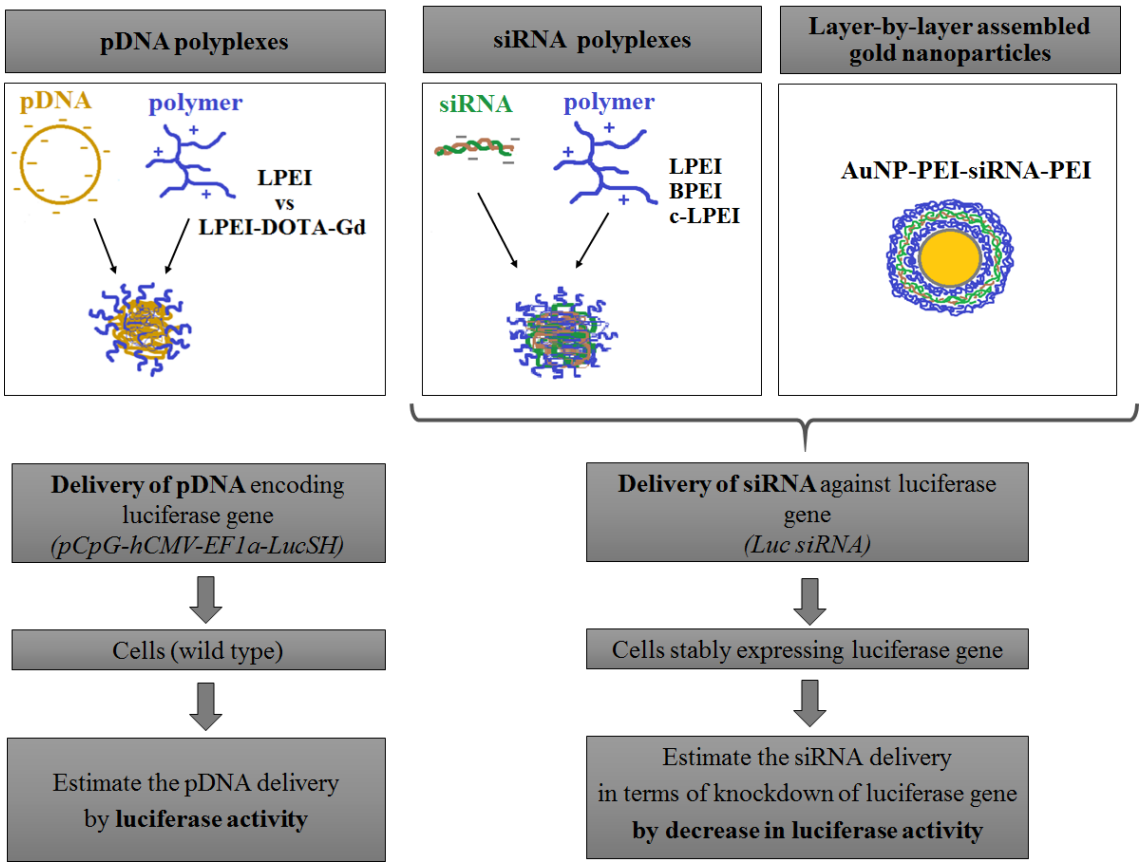

Figure 6. Scheme of the work.
pDNA = plasmid DNA, siRNA = small interfering RNA, PEI = polyethylenimine, LPEI = linear polyethylenimine, BPEI = branched polyethylenimine, c-LPEI = disulfide crosslinked linear polyethylenimine, LPEI-DOTA-Gd = linear polyethylenimine labeled with gadoteric acid, AuNP = gold nanoparticle.

3. MATERIALS AND METHODS

3.1 Technical equipment

The following technical equipment was used in this diploma thesis:

- Analytical balance (Sartorius, Goettingen, Germany)
- Biological safety cabinet (HERASAFE KS, Thermo Scientific™, Waltham, Massachusetts, USA)
- Centrifuge (HERAEUS MEGAFUGE 16R, Thermo Scientific™, Waltham, Massachusetts, USA)
- Eppendorf Research® plus pipettes (0,1-2,5 µL, 2-20 µl, 20-200 µl, 100-1000 µl) (Eppendorf, Hamburg, Germany)
- Freezer -150 °C (VWR, Radnor, Pennsylvania, USA)
- Freezer -20 °C (Allectric, Vienna, Austria)
- Incubator 37° C, 5 % CO_2 (HERACELL 150i, Thermo Scientific™, Waltham, Massachusetts, USA)
- Inverted Laboratory Microscope with LED Illumination (DM IL LED, Leica Microsystems, Wetzlar, Germany) equipped by Camera (DFC450, Leica Microsystems, Wetzlar, Germany)
- Inverted microscope (AE31 Elite Trinocular, Motic, Hong Kong, China)
- MacsQuant® Analyzer 10 (Miltenyi Biotec, Bergisch-Gladbach, Germany)
- Magnetic stirrer (MR 3001 K, Heidolph, Schwabach, Germany)
- Mini-centrifuge (Fisherbrand, Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Mr. Frosty Freezing Container (Thermo Scientific™, Waltham, Massachusetts, USA)
- Multi-Channel Pipettor (Ultra High-Performance, VWR, Radnor, Pennsylvania, USA)
- pH meter 7110 (inoLab, WTW, Weilheim, Germany)
- Pipette Controller (accu-jet® pro, Brandtech Scientific Inc., Essex, Connecticut, USA)
- Plate reader (Infinite® M200 Pro, Tecan, Männedorf, Switzerland)

- Revco™ ExF -86°C Freezer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- Refrigerator (Allectric, Veinna, Austria)
- Thermomixer (Thermomixer C, Eppendorf, Hamburg, Germany)
- Ultrapure water system (Arium®pro VF, Sartorius, Göttingen, Germany)
- Vacuboy® hand operator (Integra Biosciences, Zizers, Switzerland)
- Vacusafe™ Vacuum Aspiration System (Integra Biosciences, Zizers, Switzerland)
- Vortex mixer (ZX4, Velp Scientifica, Usmate, Italy)
- Water bath (VWB 18, VWR, Radnor, Pennsylvania, USA)

The following supplies were used in this diploma thesis:

- 0,22 µm cellulose acetate filter (VWR International, Randor, Pennsylvania, USA)
- 0,45 µm cellulose acetate filter (VWR International, Randor, Pennsylvania, USA)
- Centrifuge Tube 15 ml, 50 ml (Starlab International GmBH, Hamburg, Germany)
- Discardit™ II syringe 20 ml (Becton Dickinson S.A., Fraga, Spain)
- Eppendorf tubes (Nerbe plus GmbH, Winsen/Luhe, Germany)
- Haemocytometer: Neubauer improved (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany)
- Pipette tips (Nerbe plus GmbH, Winsen/Luhe, Germany)
- Reagent reservoirs (VWR International, West Chester, Pennsylvania, USA)
- Serological pipettes 5 ml, 10 ml, 25 ml (Sarstedt, Nümbercht, Germany)
- Storage tubes, 2D barcoded (VWR International GmBH, Vienna, Austria)
- TC Flask T25, Stand., Vent. Cap (Sarstedt, Nümbrecht, Germany)
- TC Flask T75, Stand., Vent. Cap (Sarstedt, Nümbrecht, Germany)
- Transparent 96-well flat-bottom microplate, sterile (Greiner bio-one, Frickenhausen, Germany)
- White 96-well flat-bottom microplate, transparent bottom, sterile (Greiner bio-one, Frickenhausen, Germany)

The following software was used in this diploma thesis:

- GraphPad PRISM™ version 7 (GraphPad Software Inc., La Jolla, California, USA)
- LAS X 2.0.0. (14332, Leica Microsystems, Wetzlar, Germany)
- Microsoft® Office 2010 (Microsoft, Redmond, Washington, USA)
- Tecan iControl 1.7 (Tecan, Männedorf, Switzerland)

3.2 Reagents and buffers

The following reagents were used in this diploma thesis:

- Adenosine 5′-triphosphate disodium salt trihydrate (ATP) (Cat.No. 10519979001, Roche Diagnostics, Mannheim, Germany)
- BCA Protein Assay Kit: BSA 2 mg/ml, Reagent A, Reagent B (Cat. No. 23225, Thermo Scientific, Vienna, Austria)
- Bovine serum albumin (Cat. No. 9647, Sigma- Aldrich, Steinheim, Germany)
- Cell Culture Lysis 5x Reagent (CCLR5x) (Cat. No. E153A, Promega, Mannheim, Germany)
- Coenzym A trithium salt (Cat. No. C3019, Sigma-Aldrich, Steinheim, Germany)
- D(+)-Glucose (Cat.no. 1.08337.1000, Merck, Darmstadt, Germany)
- DL-Dithiothreitol (DTT) (Cat. No. D9779, Sigma-Aldrich, Steinheim, Germany)
- D-luciferin (beetle) sodium salt (Cat. No. E464X, Promega, Mannheim, Germany)
- DMEM F12 Medium (Cat. No. D6421, Sigma-Aldrich, Steinheim, Germany)
- DMEM high glucose Medium (Cat. No. D5671, Sigma-Aldrich, Steinheim, Germany)
- Dulbecco's phosphate buffered saline (DPBS) (Cat. No. D8537, Sigma-Aldrich, Steinheim, Germany)
- Ethylenediaminetetraacetic acid (EDTA) (Cat. No. E6758, Sigma-Aldrich, Steinheim, Germany)
- Fetal bovine serum (Cat. No. F7524, Sigma-Aldrich, Steinheim, Germany)
- Glycylglycine (Cat. No. G3915, Sigma-Aldrich, Steinheim, Germany)
- Hydrochloric acid (HCl) (Cat. No. 30721, Sigma-Aldrich, Steinheim, Germany)
- L-Glutamine 200 mM (Cat. No. G7513, Sigma-Aldrich, Steinheim, Germany)

- Lipofectamine® RNAiMAX (Cat. No. 13778-150, Invitrogen, Carlsbad, California, USA)
- Magnesium Chloride 6-hydrate ($MgCl_2.6H_2O$) (Cat. No. A4425,0250, AppliChem, Darmstadt, Germany)
- N-(2-Hydroxyethyl)piperazine-N´-(2ethanesulfonic acid) (HEPES) (Cat. No. A3724, AppliChem)
- Opti-MEM® Reduced Serum Medium (Gibco)
- Passive Lysis Buffer 5x (Cat. No. E1941, Promega, Mannheim, Germany)
- Penicillin-Streptomycin (Cat. No. P0781, Sigma-Aldrich, Steinheim, Germany)
- QuantiLum® Recombinant Luciferase (Cat.No. E1701, Promega, Mannheim, Germany)
- RPMI 1640 Medium (Cat. No. R0883, Sigma-Aldrich, Steinheim, Germany)
- Sodium hydroxide (Cat. No. A6829, AppliChem, Darmstadt, Germany)
- Sodium chloride (NaCl) (Cat. No. A2942, AppliChem, Darmstadt, Germany)
- TrypLE express enzyme with phenol red (Cat. No. 12605036, Gibco, Vienna, Austria)

3.2.1 HEPES buffered saline

HBS = HEPES buffered saline (20 mM HEPES/150 mM NaCl) was prepared as follows: 1,7532 g NaCl and 0,95324 g HEPES was dissolved in 150 ml MQ-water and pH was adjusted to 7,4 using NaOH/HCl. Volume was filled up with MQ-water to 200 ml and pH was adjusted again. Solution was filtered through 0,2 µm cellulose acetate membrane syringe filter in biological safety cabinet. HBS buffer was stored at 4 °C.

3.2.2 HEPES buffered glucose

HBG = HEPES buffered glucose (20 mM HEPES/5 % (w/V) glucose) was prepared as follows: 10 g glucose and 0,95324 g HEPES was dissolved in 150 ml MQ-water and pH was adjusted to 7,4 using NaOH/HCl. Volume was filled up with MQ-water to 200 ml and pH was adjusted again. Solution was filtered through 0,2 µm cellulose acetate membrane syringe filter in biological safety cabinet. HBG buffer was stored at 4 °C.

3.2.3 Luciferase Assay Buffer

For reading the firefly luciferase based assay, Luciferase Assay Buffer (LAB) was prepared, consisting of Luciferin mixed with Luciferin Buffer (LB). Luciferin is the substrate in principal reaction of firefly luciferase assay described in section 3.7.2. Composition of LB, in accordance with protocol from University of Birmingham (Dash, University of Birmingham), represents appropriate environment for the reaction including essential factors such as ATP, Mg^{2+} etc. (Marques and Esteves da Silva 2009) and its employment in firefly luciferase assay was optimized previously as described in diploma thesis of Katharina Müller (2017, University of Vienna).

3.2.3.1 **Stock solutions for Luciferase Assay Buffer preparation**

Preparation of 1 M Glycylglycine solution

Solution was prepared fresh at day of preparation of each batch of LB. 2,6424 g Glycylglycine was dissolved in approximately 18 ml MQ-water and pH 8 was adjusted using NaOH. Volume was filled up to 20 ml with MQ-water, solution was passed through 0,45 µm cellulose acetate membrane syringe filter and stored at 4 °C.

Preparation of 100 mM $MgCl_2$ solution

1,065 g of $MgCl_2$ was dissolved in 50 ml of MQ-water. Solution was passed through 0,45 µm cellulose acetate membrane syringe filter and stored at room temperature.

Preparation of 500 mM EDTA solution

7,306 g EDTA in approximately 30 ml MQ-water was stirred for few minutes resulting in white suspension with acidic pH. To avoid precipitation of EDTA, pH change was done quickly by adding 7 times 1 ml of 10 M NaOH while stirring solution and subsequently pH was adjusted to 8 using NaOH. Volume was filled up to 50 ml and pH was adjusted again using NaOH or HCl. To dissolve EDTA properly, mixture was stirred for 30 – 40 min resulting in solution which was passed through 0,45 µm cellulose acetate membrane syringe filter and stored at 4 °C.

Preparation of Coenzyme A 42,6 mg/ml solution

Preparation was carried out with respect to coenzyme A temperature sensitivity. 100 mg of Coenzyme A was dissolved in 2347 µl precooled MQ-water (4 °C) Solution was either used within 1 h to prepare the buffer and stored on ice or stored in 250 µl aliquots at -80 °C and thawed on ice for preparation of the buffer.

Preparation of 10 mM Luciferin solution

The solution was a kind gift from Katharina Müller (MMCT, University of Vienna). In brief, for preparation of 48 ml solution, 144.3 mg of Luciferin powder, 1,41 ml of fresh 1 M Glycylglycine and MQ-water up to 48 ml was used and pH 8,0 was adjusted. Preparation and any later handling the solution must be done under light protection. The solution can be either immediately used for LAB preparation or stored at -80°C.

3.2.3.2 **Preparation of Luciferase Assay Buffer**

Before starting the preparation, sufficient amount of MQ-water was precooled at 4°C. Coenzyme A solution, Glycylglycine solution, DTT and ATP were handled with respect to their temperature sensitivity including keeping on ice, precooling tubes and using precooled MQ-water if dissolving. Composition of LB and LAB, respectively, is described in Table 1. Reagents were added in following sequence: Firstly, 1M Glycylglycine, 100 mM $MgCl_2$ and 500 mM EDTA were added. Then ATP and DTT were dissolved in MQ-water and added to mixture. Subsequently, Coenzyme A 42,6 mg/ml solution was added and final volume was filled up with precooled MQ-water. Finally, pH 8,2 was adjusted using 1 M NaOH and buffer was passed through 0,2 µm cellulose acetate membrane syringe filter in biological safety cabinet. Obtained LB can be either immediately used for LAB preparation or stored at -80°C. Mixing of LB and 10 mM Luciferin solution resulting in LAB was done under light protection.

Table 1. Composition of Luciferase Assay Buffer.

Reagent:	*Amount for preparation of 50 ml Luciferin Buffer*	
1 M Glycylglycine	1 ml	**Luciferin Buffer = LB** / **Luciferase Assay Buffer = LAB**
100 mM $MgCl_2$	0,5 ml	
500 mM EDTA	10 µl	
DTT	25,4 mg	
ATP	13,9 mg	
Coenzyme A 42,6 mg/ml	0,25 ml	
MilliQ-water	up to 50 ml	
1 M NaOH	for pH 8,2 adjustment	
	Amount added to prepared 50 ml Luciferin Buffer:	
10 mM Luciferin solution	2,5 ml	

3.3 Cell culture

3.3.1 Cell lines used for gene delivery experiments

A549, human lung adenocarcinoma cells, were kind gift from Dr. Johannes Winkler (University of Vienna), originating from the European Collection of Authenticated Cell Cultures (ECACC). They were grown in RPMI 1640 media supplemented with 10 % (V/V) 0,1 µm filtered FBS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were used for transfection experiments between passage 98 and 101.

CT26, murine colon carcinoma cells, were kind gift from Prof. Walter Berger (Medical University of Vienna). These cells were grown in DMEM F12 media supplemented with 10 % (V/V) 0,1 µm filtered FBS, 2,5 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin Cells were used for transfection experiments between passage 28 and 44.

3.3.2 Cell lines used for gene knockdown experiments

Cell lines stably expressing firefly luciferase gene were used for gene knockdown experiments.

MDA-MB-231 (wild type), human breast adenocarcinoma cells derived from metastatic pleural effusion, and HeLa (wild type), human cervical adenocarcinoma cells, were purchased from American Type Culture Collection (ATCC) and transduced lentivirally with PGK-eGFP-Luc by M. Sc. Julia Maier (MMCT, University of Vienna). The cells were sorted by flow cytometry to culture only the transduced cells. Obtained MDA-MB-231-PGK-eGFP-Luc cells and HeLa-PGK-eGPF-Luc cells were maintained in DMEM high glucose media supplemented with 10 % (V/V) 0,1 µm filtered FBS, 4 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. MDA-MB-231-PGK-eGPF-Luc cells were used for experiments between passage 58 and 88. HeLa-PGK-eGFP-Luc cells were used for experiments between passage 58 and 66.

3.3.3 Cell culture work

All cell culture work was carried out under aseptic conditions in biological safety cabinet. All cell lines were thawed at least 2 weeks before performing experiment, maintained in T75 cell culture flask at 37 °C in a humidified atmosphere containing 5 % CO_2. Cells

were regularly observed in microscope to assess confluency and check for contamination or morphological changes. Cell culture media was regularly changed not to maintain cells more than 3 days in same media and cells were splitted every 3 or 4 days performing following steps: Cell culture media was removed, cells were gently washed with DPBS to remove dead cells and traces of media, 1 ml of TrypLE (3 ml in case of A549) was added and flask was incubated for 4 min in 37 °C. Flask was tapped with hands if needed. Detached cells were flushed twice with 3 ml and 4 ml of cell culture media, transferred in 15 ml centrifuge tube and centrifuged for 5 min at 200 x g. Supernatant was removed and pellet was resuspended in 1 ml of cell culture media. From this cell suspension, needed amount according to splitting ratio was added in 12 ml of cell culture media in flask.

All fluids added to cells were prewarmed to 37 °C. For handling media 50 ml aliquots were made to avoid multiple prewarming cycles.

3.3.4 Cell seeding

Cells were seeded in 96-well plate. Firstly, cell culture media was added per well to have final volume in well 200 µl. Then counted amount (as described below) of cell dilution was added per well. Plate was regularly gently mixed to ensure appropriate cell distribution in wells. After seeding each well of plate was checked under microscope and cells were incubated overnight (37 °C, 5 % CO_2).

Cell counting based on haemocytometer

After cell splitting procedure a cell dilution (for cell counting and cell seeding) in cell culture media was made from cell suspension. For accurate seeding, dilution factor was chosen not to seed less than 10 µl of cell dilution per well and to have 100-200 cells in each counting square. 10 µl of this dilution was transferred in Neubauer chamber. 5 squares were counted and cell density (cells/ml) in cell suspension was assessed using following equation:

$$\text{cell density} = \frac{\text{number of cells counted}}{\text{number of squares counted}} * \text{dilution factor} * 10{,}000$$

Volume of cell dilution to be added per well was counted:

$$\mu\text{l of cell dilution} = \frac{1000 * \text{seeding number}}{\text{cell density in cell suspension}} * \text{dilution factor}$$

Cell counting based on flow cytometry

After cell splitting procedure 1:10 dilution (for cell counting) in DPBS was made from cell suspension. This dilution was counted using MacsQuant® Analyzer 10 (Miltenyi Biotec, Bergisch-Gladbach, Germany). Original cell density in cell suspension was counted and cell suspension was diluted in cell culture media accordingly (resulting in cell dilution for cell seeding). Volume of cell dilution to be added per well was counted as described above. Optimally, dilution factor was chosen not to seed less than 10 µl of cell dilution per well.

3.4 Synthesis of nanoparticles for DNA and siRNA delivery

Special materials:

- Branched polyethylenimine (BPEI) of 25 kDa (Cat.No. 408727-100ML, Sigma-Aldrich, Steinheim, Germany)
- Linear polyethylenimine (LPEI) of 10 kDa and LPEI-DOTA-Gd in ratios 1:5, 1:15 and 1:25 were kind gift from Mag. Alexander Taschauer (MMCT, University of Vienna)
- Crosslinked LPEI (c-LPEI) was kind gift from Hermann Bloβ. (MMCT, University of Vienna) and was crosslinked from LPEI of 3 kDa using as the linker Lomant's reagent (3'-dithiopropionic acid di-(*N*-succinimidylester)) with molar ratio polymer:linker 2:1
- siRNA targeted against firefly luciferase gene (LucsiRNA) and negative control non-targeted siRNA (NCsiRNA) were provided under Innovative Medicines Initiative Joint Undertaking COMPACT
- pCpG-hCMV-EF1α-LucSH plasmid DNA is described in (Magnusson et al. 2011)

3.4.1 Polyplex formation

Composition of polyplexes in all experiments is characterized by N/P ratio (molar ratio of nitrogens from the polyethylenimine (PEI) and phospates from nucleic acid) which is calculated as follows:

$$\mathrm{N/P\ ratio} = \frac{m_{(PEI)}/M_{w_r N}}{m_{(NA)}/M_{w_r P}}$$

where $M_{w_r N}$ = molecular weight of PEI per nitrogen (= 43 g/mol), $M_{w_r P}$ = molecular weight of nucleic acid per phosphate (= 330 g/mol), $m_{(PEI)}$ = mass of PEI (μg); $m_{(NA)}$ = mass of nucleic acid (μg).

Mass of nucleic acid was calculated from required amount of nucleic acid per well. In case of pDNA transfection 200 ng per well was used. In case of siRNA 1 pmol, 5 pmol, 10 pmol or 36 pmol per well were used and mass of siRNA was calculated as follows:

$$m_{(siRNA)} = M_{(siRNA)} * n_{(\mathrm{siRNA})}$$

where $m_{(siRNA)}$ = mass of siRNA; $M_{(siRNA)}$ = 0,01385 g/mol; $n_{(\mathrm{siRNA})}$ = 1 pmol, 5 pmol, 10 pmol or 36 pmol.

Firstly, nucleic acid and PEI were separately diluted in HBS or HBG. siRNA dilutions were finished right before polyplexing. Volume of nucleic acid taken from its stock and total volume of nucleic acid dilution was calculated as follows:

$$V_{(\mathrm{NA})} = \frac{m_{(\mathrm{NA})}}{C_{O\,(\mathrm{NA})}}$$

$$V_{1\,(\mathrm{NA})} = \frac{m_{(\mathrm{NA})}}{2 * C_1} * 1000$$

where $V_{(\mathrm{NA})}$ = volume of nucleic acid taken from stock (µl), $C_{O\,(\mathrm{NA})}$ = concentration of nucleic acid stock (µg/µl), $V_{1\,(\mathrm{NA})}$= final volume of nucleic acid dilution (µl), C_1= final concentration of nucleic acid for polyplexing (µg/ml). This $C_1$was kept 20 µg/ml when forming pDNA polyplexes. For siRNA polyplexes was C_1 kept either 2 µg/ml for experiments with 1 – 10 pmol siRNA/well or 20 µg/ml for experiments with 36 pmol/well.

As polyplexes are prepared by mixing equal volumes of PEI and nucleic acid dilutions, total volume of PEI dilution $V_{1\,(\mathrm{PEI})}$= $V_{1\,(\mathrm{NA})}$. Amount of PEI and volume of PEI taken from its stock was calculated as follows:

$$m_{(\mathrm{PEI})} = \frac{m_{(\mathrm{NA})} * \mathrm{N/P\ ratio} * M_{w_r N}}{M_{w_r P}}$$

$$V_{(\mathrm{PEI})} = \frac{m_{(\mathrm{PEI})}}{C_{0\,(\mathrm{PEI})}}$$

where $V_{(\mathrm{PEI})}$= volume of PEI taken from stock (µl), $C_{0\,(\mathrm{PEI})}$ = concentration of PEI stock (µg/µl).

Polyplexes were created by adding PEI dilution to nucleic acid dilution and mixing by flash pipetting. After mixing polyplexes were incubated at room temperature for *t* = 40 min, *t* = 30 min or *t* = 0 min, where *t* = incubation time after polyplexing.

3.4.2 Layer-by-Layer assembled gold nanoparticles

Gold nanoparticles for siRNA delivery were generated by Hermann Bloß (MMCT, University of Vienna) and were coated by Layer-by-Layer assembly as described by Elbakry et al. (2009). Briefly, as seen in Figure 7, gold nanoparticles represent a core which is coated by different oppositely charged layers. As Layer 1 positively charged polyethylenimine was used (either BPEI or c-LPEI), Layer 2 is negatively charged siRNA and as terminal Layer 3 nanoparticles bear another layer of BPEI or c-LPEI.

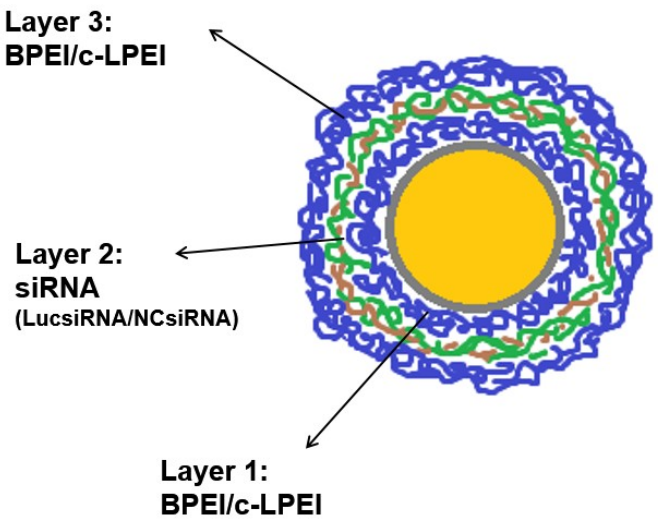

Figure 7. Composition of Layer-by-Layer assembled gold nanoparticles.
Layer 1 is BPEI or c-LPEI, Layer 2 is represented by siRNA and as terminal Layer 3 nanoparticles bear another layer of BPEI or c-LPEI.

3.4.3 Lipofectamine® RNAiMAX based lipoplexes

1,5 µl Lipofectamine® RNAiMAX Reagent was diluted in 23,5 µl Opti-MEM® Media to reach total volume 25 µl. Volume of siRNA equal to 5 pmol was diluted in Opti-MEM® Media with total volume 25 µl. Each dilution was mixed and siRNA dilution was added to diluted Lipofectamine® RNAiMAX Reagent at ratio 1:1, mixed by flash pipetting and incubated for 5 min at room temperature. Subsequently, 10 µl was added per well in triplicates.

Same procedure was performed using LucsiRNA and NCsiRNA simultaneously.

3.5 *In vitro* studies with nanoparticles for pDNA delivery

A549 or CT26 cells were seeded (10,000 cells/well) in white 96-well plate and incubated (37 °C, 5 % CO_2) overnight. The seeding density 10,000 of A549 cells/well for *in vitro* transfection studies was optimized previously as described by Taschauer et al. (2016) and the seeding density of CT26 was kept the same. Seeding was done based on cell counting using haemocytometer for CT26 cells and flow cytometry for A549 cells depending on availability of the equipment for the counting method.

Before transfection, the cell culture media was replaced by 90 µl of basal media. Polyplexes were created in HBS or HBG as described above using pCpG-hCMV-EF1α-LucSH plasmid DNA and LPEI 10 kDa, LPEI-DOTA-Gd 1/5, LPEI-DOTA-Gd 1/15 or LPEI-DOTA-Gd 1/25 at N/P ratio 6 or 9. Immediately after polyplexing (t = 0 min), 10 ul of created polyplexes was added to the cells so the final concentration 200 ng pDNA/well was obtained. Plate was incubated at 37 °C, 5 % CO_2 for 4 h, afterwards different procedure was followed for 4 h and 24 h treatment. In case of 4 h treatment, the media from each well was aspirated, 200 µl of complete cell culture media was added per well and plate was incubated for 20 h. In case of 24 h treatment, the present content in wells was kept, 100 µl of complete cell culture media was added per well to provide nutrients and other essential substances for cells growth and plate was incubated for 20 h.

For each experiment, untreated cells were kept under the same conditions as samples: At time of transfection of samples cell culture media was replaced by 100 µl of basal media and plate was incubated at 37 °C, 5 % CO_2 for 4 h Afterwards media was replaced by 200 µl of complete cell culture media (= untreated wells for 4 h incubation) or 100 µl of complete cell culture media was added to present content in wells (= untreated wells for 24 h incubation) and plate was incubated for 20 h.

After 24 h post transfection the media was aspirated, cells were washed with DPBS and treated with 30 µl passive lysis buffer. Luciferase activity in 10 µl cell lysate and protein concentration in 20 µl cell lysate were measured performing firefly luciferase expression assay and BCA assay, respectively, as described in section 3.7.

3.6 *In vitro* studies with nanoparticles for siRNA delivery

3.6.1 Seeding density optimization for siRNA delivery experiments

Seeding density optimization based on BCA and firefly luciferase assay

MDA-MB-231-PGK-eGFP-Luc were seeded in white 96-well plate in triplicates at different seeding numbers: 5,000, 10,000, 20,000, 40,000 and 80,000 cells/well and incubated overnight.

24 h after seeding the media was aspirated, cells were washed with DPBS and treated with 30 µl passive lysis buffer. Luciferase activity in 10 µl cell lysate and protein concentration in 20 µl cell lysate were measured performing firefly luciferase expression assay and BCA assay, respectively, as described in section 3.7.

Seeding density optimization based on confluency assessment

20,000 cells/well and 30,000 cells/well of MDA-MB-231-PGK-eGFP-Luc were seeded in white 96-well plate and incubated overnight. 24 h after seeding, cell density was documented using Inverted Laboratory Microscope with LED Illumination (DM IL LED, Leica Microsystems, Wetzlar, Germany) equipped by Camera (DFC450, Leica Microsystems, Wetzlar, Germany).

3.6.2 Polyplexes mediated siRNA delivery

Cells stably expressing firefly luciferase gene were seeded in white 96-well plate: HeLa-PGK-eGFP-Luc (20,000 cells/well) and MDA-MB-231-PGK-eGFP-Luc (20,000 cells/well or 30,000 cells/well) and incubated (37 °C, 5 % CO_2) overnight. The seeding density of MDA-MB-231-PGK-eGPF-Luc cells was optimized as described in sections 3.6.1 and 4.3.1. Inspired by the optimization together with considering shorter doubling time of HeLa-PGK-eGFP-Luc cells, also the seeding density of HeLa-PGK-eGFP-Luc was chosen. Seeding was done based on cell counting using haemocytometer.

According to incubation and polyplex parameters (Table 2 and Table 3) transfection was started as follows: Cell culture media was replaced by calculated amount of basal or complete media. Polyplexes were generated as described in section 3.4.1. using LPEI, BPEI or c-LPEI and siRNA. Polyplexes tested for knockdown composed of LPEI and siRNA against luciferase gene (LucsiRNA). For each polyplex at each parameter

negative control was kept composed of LPEI and negative control siRNA (NCsiRNA) with scrambled sequence which will not knockdown the luciferase gene. Polyplexes were added to the cells immediately after generating (t = 0 min) or after t = 30 min or t = 40 min. Plate was incubated for 48 h at 37 °C, 5 % CO_2 with or without changing or replacing the media after 4 h post transfection.

Untreated cells were kept for each experiment at same incubation conditions as samples.

In experiments with BPEI/siRNA and c-LPEI/siRNA polyplexes, Lipofectamine® RNAiMAX transfection procedure was included as positive control. The procedure was performed using the same batch of siRNA as used in particular experiment. Generation of Lipofectamine® RNAiMAX based lipoplexes is described in section 3.4.3. Independently on incubation conditions tested in particular experiment, 48 h incubation in complete media was performed for Lipofectamine® RNAiMAX transfection procedure as follows: Cell culture media was replaced by 190 µl of complete media and 10 µl of Lipofectamine® RNAiMAX based lipoplexes were added. Plate was incubated for 48 h at 37 °C, 5 % CO_2.

After 48 h post transfection the media was aspirated, cells were washed with DPBS and treated with 30 µl passive lysis buffer. Decrease of luciferase activity in 10 µl cell lysate and protein concentration in 20 µl cell lysate were measured performing firefly luciferase expression assay and BCA assay, respectively, as described in section 3.7.

Table 2. List of parameters for optimization of PEI/siNA polyplexes mediated siRNA delivery.

	Type of PEI		
	LPEI	**BPEI**	**c-LPEI**
:Parameters:	*Cell line*: MDA-MB-231-PGK-eGFP-Luc (20,000 cells/well and 30,000 cells/well)	*Cell line:* MDA-MB-231-PGK-eGFP-Luc (30,000 cells/well) HeLa-PGK-eGFP-Luc (20,000 cells/well)	*Cell line:* MDA-MB-231-PGK-eGFP-Luc (30,000 cells/well) HeLa-PGK-eGFP-Luc (20,000 cells/well)
	Incubation parameters: **4hB:** 4 h in basal media + replacing with complete media **4hC:** 4 h in complete media + replacing with complete media **48hC**: 48 h in complete media	*Incubation parameters:* **4hB:** 4 h in basal media + replacing with complete media **4hC:** 4 h in complete media + replacing with complete media **4hB+44hC:** 4 h in basal media + adding complete media **48hC**:48 h in complete media	*Incubation parameters:* **4hB+44hC:** 4 h in basal media + adding complete media **48hC**: 48 h in complete media
	Polyplex parameters N/P ratios: 6, 9, 20 HBS/HBG t = 0 min, 30 min Amount of siRNA/well: 5 pmol	*Polyplex parameters* N/P ratios: 3, 6, 9, 20 HBS/HBG t = 0 min, 40 min Amount of siRNA/well: 1 pmol 5 pmol 10 pmol 36 pmol	*Polyplex parameters* N/P ratios: 6, 9, 20 HBS/HBG t = 0 min, 40 min Amount of siRNA/well: 5 pmol 10 pmol 36 pmol

Table 3. Detailed list of incubation parameters for siRNA delivery.

Name	Description of incubation parameters
4hB(I)	4 h in basal media (200 µl in total), then replaced by 200 µl of complete media with ATB and incubated for 44h
4hB(II)	4 h in basal media (100 µl in total), then replaced by 200 µl of complete media without ATB and incubated for 44h
4hC	4 h in complete media with ATB (200 µl in total), then replaced by 200 µl of complete media with ATB and incubated for 44h
4hB+44hC	4 h in basal media (100 µl in total), then adding 100 µl of complete without ATB and incubated for 44h
48hC(I)	48 h in complete media with ATB (200 µl in total)
48hC(II)	48 h in complete media without ATB (200 µl in total)
48hC(III)	48 h in complete media without ATB + additional HBG/HBS* (200 µl in total)

* Volume of polyplexes in HBS/HBG added to wells differs among different siRNA amounts/well. For this reason, missing volume of HBS/HBG was added to respective wells to keep same volume of HBS/HBG among wells treated with polyplexes.

3.6.3 Layer-by-Layer assembled gold nanoparticles mediated siRNA delivery

MDA-MB-231-PGK-eGFP-Luc (20,000 cells/well or 30,000 cells/well) cells stably expressing firefly luciferase gene were seeded in white 96-well plate: and incubated (37 °C, 5 % CO_2) overnight. Seeding was done based on cell counting using haemocytometer.

Cell culture media was replaced by calculated amount of complete media with or without antibiotics to gain total volume in well 200 µl. Layer-by-Layer assembled gold nanoparticles were added to wells in different concentrations expressed as particles/well based on nanoparticles tracking analysis (NTA) measurements of the nanoparticles suspension performed by Hermann Bloß (MMCT, University of Vienna). Nanoparticles carrying either targeted LucsiRNA (tested for gene silencing efficiency) or siRNA with scrambled sequence (which will not knockdown the luciferase gene) NCsiRNA (as negative control) were applied. Plate was incubated for 48 h at 37 °C, 5 % CO_2.

Untreated cells were kept for each experiment at same incubation conditions as samples.

If positive control was included, Lipofectamine® RNAiMAX transfection procedure was performed. Generation of Lipofectamine® RNAiMAX based lipoplexes is described in section 3.4.3. Independently on incubation conditions tested in particular experiment, 48 h incubation in complete media was performed for Lipofectamine® RNAiMAX transfection procedure as described previously in in section 3.6.2.

After 48 h post transfection media was aspirated, cells were washed with DPBS and treated with 30 µl passive lysis buffer. Decrease of luciferase activity in 10 µl cell lysate and protein concentration in 20 µl cell lysate were measured performing firefly luciferase expression assay and BCA assay, respectively, as described in section 3.7.

3.7 Reporter gene expression assay

3.7.1 Cell lysis

Media was removed from wells and cells were washed once with 200 µl DPBS. Passive lysis buffer 1x (PLB 1x) was prepared 30 min in advance using PLB 5x and MQ-water. 30 µl of PLB 1x was added to washed cells and to wells serving as blank for samples (triplicate) and plate was shaken for 30 min at 500 rpm, room temperature. Lysis was checked in microscope, lysate was mixed, 20 µl was transferred in transparent 96-well plate for performing BCA assay and firefly luciferase based assay was performed with 10 µl of lysate.

3.7.2 Firefly luciferase based assay

Firefly (*Photinus pyralis*) luciferase is enzyme which catalyzes the reaction of substrate D-luciferin resulting in excited state product oxyluciferin emitting green luminescent light while returning to the ground state (Marques and Esteves da Silva 2009).

In case of pDNA transfection, the firefly luciferase gene was encoded in a plasmid (pCpG-hCMV-EF1α-LucSH) delivered to the cells, serving as reporter gene. Expression of luciferase protein and thus its luciferase activity indicates the transfection efficiency.

For siRNA delivery, cells stably expressing firefly luciferase were used. Effective delivery of siRNA against firefly luciferase (LucsiRNA) results in knockdown of luciferase expression and decrease of luciferase activity.

10 µl of cell lysate as well as 10 ul of blank for samples was used for measuring the luminesce after injecting LAB by plate reader (Infinite® M200 Pro, Tecan, Männedorf, Switzerland) at following settings:

- Injection volume of substrate: 100 µl
- Speed: 200 µl/sec.
- Refill speed: 100 µl/sec.
- Injection Refill Volume: 500 µl
- Wait time: 2 sec.
- Integration time: 10 000 ms
- Settle time: 0 ms

3.7.3 BCA assay

Luminescence measurement was normalized for protein concentration of the samples. This was determined performing BCA assay which is based on forming intense purple 2:1 complex of bicinchoninic acid (BCA) and cuprous ion (Cu^{1+}). Cu^{1+} is produced by reduction of Cu^{2+} by proteins in alkaline environment. Final purple complex is a chromophore with an absorbance maximum at 562 nm (Smith et al. 1985).

Protein concentration in samples can be determined using a calculation based on standard curve. This calibration was done for each experiment to ensure that standard dilutions undergo exactly same conditions as samples. Standard dilutions of bovine serum albumin (BSA) were prepared according to Table 4. Firstly, PLB 2x was prepared from PLB5x and MQ-water and let equilibrate for 30 minutes. Then, standard dilutions were prepared in following sequence: MQ-water, BSA 2 mg/ml, PLB 2x. Dilutions were mixed and 25 µl was pipetted in triplicates in transparent 96-well plate.

Table 4. Standard dilutions of bovine serum albumin for BCA assay.

BSA (µg/ well)	MQ-water (µl)	BSA 2 mg/ml (µl)	PLB 2x (µl)
25	0	50.0	50.0
18.75	12.48	37.52	50.0
12.5	25.0	25.0	50.0
6.25	37.52	12.5	50.0
3.125	43.76	6.24	50.0
0.625	48.76	1.252	50.0
Blank	50.0	0	50.0

Samples for BCA assay were prepared as follows: Cells were lysed as described in section 3.7.1., 20 µl of cell lysate as well as 20 µl of blank for samples was transferred in transparent 96-well plate. To reach same volume as standard dilutions 5 µl of MQ-water was added per well.

Working reagent for BCA assay was prepared by mixing 50 parts of Reagent A and 1 part of Reagent B and 200 µl was quickly added per well to standards and samples including blanks. Plate was shaken for 30 sec at 300 rpm, room temperature; then incubated at 37 °C for 30 min. After finishing incubation, plate was cooled to room temperature for 10 min and absorbance was measured at plate reader (Infinite® M200 Pro, Tecan, Männedorf, Switzerland) at following settings:

- wavelength: 562 nm
- bandwidth: 9 nm
- number of flashes: 25
- settle time: 0 ms

3.7.4 Luciferase based assay with recombinant Firefly luciferase

To test each batch of LAB, luciferase based assay with recombinant Firefly luciferase was performed. Recombinant Firefly luciferase stocks were handled with respect to its temperature sensitivity. Previously, stock B representing recombinant Firefly luciferase

at concentration 1 µg/µl in Cell Culture Lysis 1x Reagent (1x CCLR) was prepared and stored at -80 °C. For less concentrated dilutions, diluent 1x CCLR with BSA 1 mg/ml was prepared by mixing 1 part of 5x CCLR and 4 parts of BSA 1,25 mg/ml. BSA as exogenous protein serves to stabilize luciferase enzyme at extremely dilute concentrations (Promega, 2016). Subsequently, stock C was prepared as 1:10 dilution of stock B in 1x CCLR with BSA 1 mg/ml resulting in final concentration of recombinant Firefly luciferase 100 ng/µl.

According to Table 5, serial dilutions of recombinant Firefly luciferase and blank were prepared and 10 µl per well was pipetted in triplicates in white 96-well plate and used for measuring the luminesce after injecting LAB by plate reader (Infinite® 200Pro, Tecan, Männedrof, Switzerland) at settings described in section 3.7.2.

Table 5. Dilutions of recombinant Firefly luciferase.

Dilution ID	**Volume from Stock C**	**1x CCLR with BSA 1 mg/ml**	**Final Volume**	**Concentration**	**Volume used per well**	**Amount of enzyme per well**
1	5 µl	45 µl	50 µl	10 ng/µl	10 µl	100 ng
1:10 serial dilutions	**Volume taken**	**1x CCLR with BSA 1 mg/ml**	**Final Volume**	**Concentration**	**Volume used per well**	**Amount of enzyme per well**
2	5 µl from 1	45 µl	50 µl	1 ng/µl	10 µl	10 ng
3	5 µl from 2	45 µl	50 µl	0,1 ng/µl	10 µl	1 ng
4	5 µl from 3	45 µl	50 µl	0,01 ng/µl	10 µl	0,1 ng
5	5 µl from 4	45 µl	50 µl	0,001 ng/µl (= 1 pg/µl)	10 µl	0,01 ng
6	5 µl from 5	45 µl	50 µl	0,0001 ng/µl (= 0,1 pg/µl)	10 µl	0,001 ng (=1 pg)
BLANK	-	50 µl	50 µl	0 ng/µl	10 µl	0 ng

3.8 Data analysis

All experiments were performed in triplicates.

As firefly luciferase activity was measured in 10 µl of lysate, for further data processing Relative light units (RLU) per 30 µl lysate (RLU/well) were calculated.

As protein amount was measured in 20 µl lysate, for further data processing protein amount per 30 µl lysate (µg/well) was calculated.

If one experiment was carried out, the data are shown as mean values of triplicates ± SD. If two or three experiments were carried out, the data are shown as mean values of two/three independent experiments ± SD. Since the present work focuses predominantly on optimization of parameters, for this reason mostly only one or two independent experiments were carried out under the same conditions. Therefore, no statistical analysis was applied on presented results.

3.8.1 Different methods of data analysis for siRNA delivery experiments

In method 1 the data are shown as %RLU of untreated cells (UT). This is calculated as follows:

$$\frac{\text{RLU sample}}{\text{RLU untreated}} \text{ x } 100$$

In method 2 BCA normalization is used and the data are shown as: %RLU/µg of untreated cells (UT) which is calculated as follows:

$$\frac{\text{RLU sample/µg sample}}{\text{RLU untreated/µg untreated}} \text{ x } 100$$

As each formulation was tested using targeted LucsiRNA and also negative control NCsiRNA it can be assumed that the negative control formulation has hypothetically same toxicity as the formulation containing LucsiRNA. In method 3 are shown %RLU of negative control (negCTRL) which is calculated as follows:

$$\frac{\text{RLU sample}}{\text{RLU negCTRL}} \text{ x } 100$$

4. RESULTS

4.1 Testing housemade Luciferase Assay Buffer with recombinant luciferase

As described in section 3.7.4, each batch of housemade Luciferase Assay Buffer (LAB) was tested by luciferase based assay with recombinant Firefly luciferase protein. In this thesis, three different batches of LAB were used with one batch tested previously (Katharina Müller, Diploma Thesis 2017, University of Vienna). Testing of two other batches is shown in Figure 8 with comparable results for concentrations of recombinant Firefly luciferase higher than 0,1 ng/well and Relative light units (RLU) higher than 10^4, respectively.

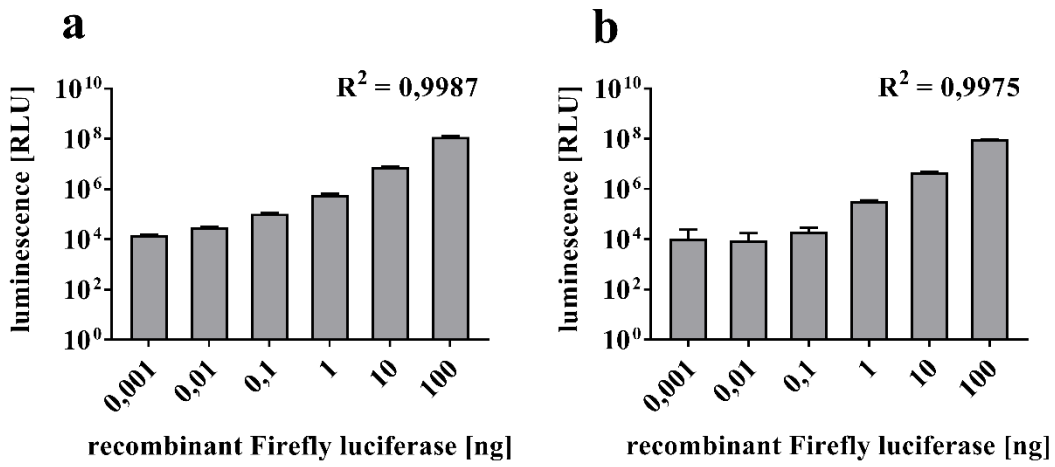

Figure 8. Testing housemade Luciferase Assay Buffer with recombinant luciferase.
Serial dilutions of recombinant Firefly luciferase were used for measuring the luminescence after injecting housemade Luciferase Assay Buffer of two different batches (a and b). Mean values of triplicates ± SD are shown.

4.2 Nanoparticles mediated gene delivery studies

4.2.1 Effect of DOTA-Gd labelling on transfection efficiency of LPEI-DOTA-Gd

To compare transfection efficiency of newly synthetized LPEI-DOTA-Gd to LPEI, CT26 cells were incubated (as described in section 3.5), for 4 h and 24 h with polyplexes composed of either LPEI or LPEI-DOTA-Gd 1/5, LPEI-DOTA-Gd 1/15, LPEI-DOTA-Gd 1/25 and pCpG-hCMV-EF1α-LucSH plasmid DNA encoding Firefly luciferase gene at final concentration 200 ng pDNA/well. Polyplexes generated in HBS or HBG and at N/P 6 or N/P 9 were compared. As seen in Figure 9, for all types of polyplexes shows N/P 9 better transfection efficiency than N/P 6 at 4 h treatment. At 24 h treatment, even no or negligible transfection by polyplexes generated at N/P 6 was also observed which could be due to lower N/P ratio and thereby inefficient delivery of pDNA. Therefore, for comparison of LPEI-DOTA-Gd and LPEI transfection efficiency, polyplexes generated at N/P 9 were chosen. LPEI-DOTA-Gd (all 1/5, 1/15 and 1/25) showed similar transfection efficiency as LPEI for both 4 h and 24 h treatment as well as for HBS and HBG.

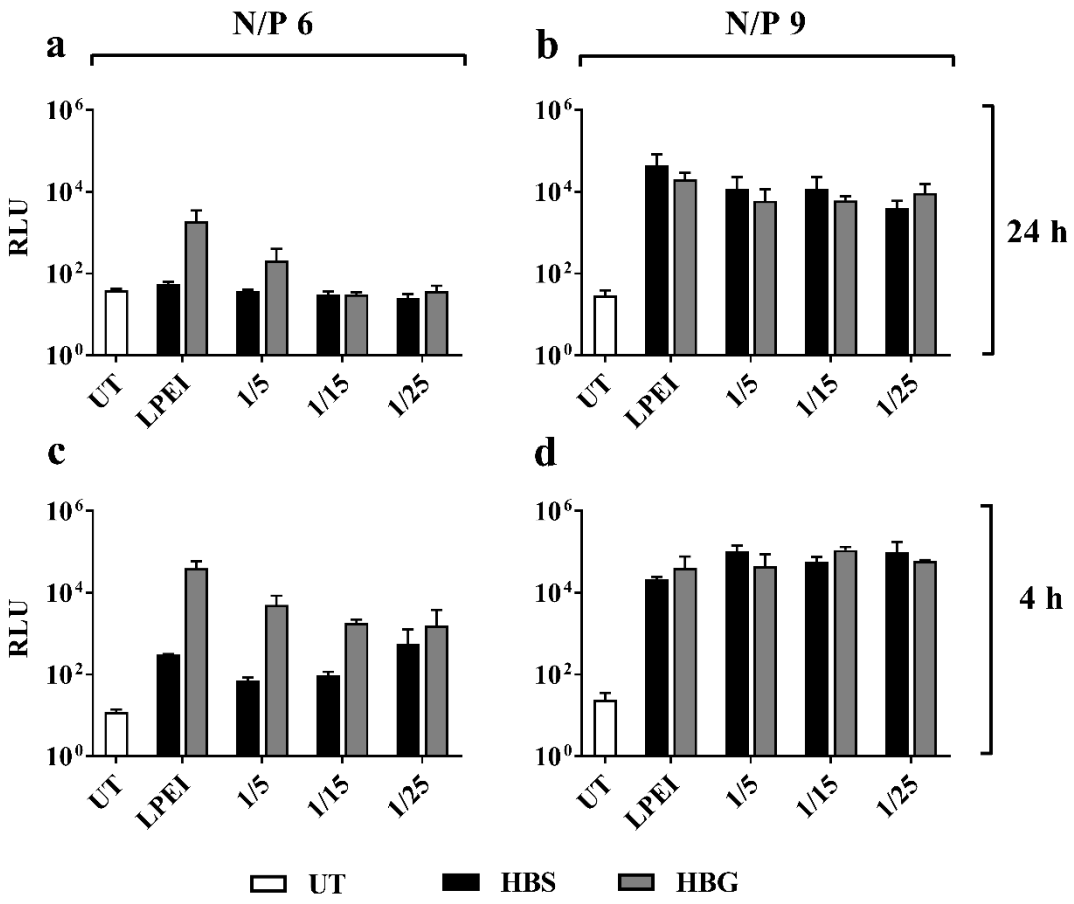

Figure 9. Transfection efficiency of LPEI-DOTA-Gd-based polyplexes compared to LPEI-based polyplexes tested on CT26 cells.

CT26 cells were treated for 24 h (a, b) or 4 h (c, d) with LPEI- and LPEI-DOTA-Gd (1/5, 1/15, 1/25)-based polyplexes generated at N/P 6 (a, c) or N/P 9 (b, d) in 20 mM HEPES/150 mM NaCl (HBS) or in 20 mM HEPES/5 % (w/V) glucose (HBG) with pDNA encoding firefly luciferase gene (pCpG-hCMV-EF1α-LucSH) with final concentration 200 ng pDNA/well or were untreated (UT). 24 h after starting the treatment luciferase activity was measured. Data are shown as RLUs per well without normalization by protein amount. a, c: mean values of triplicates ± SD are shown. b, d: mean values of two independent experiments ± SD are shown.

To confirm this result, 24 h treatment was repeated in three independent experiments with A549 cells using polyplexes generated at N/P 9 in HBS or HBG. As shown in Figure 10, LPEI-DOTA-Gd polyplexes created in HBG had similar transfection efficiency as LPEI. LPEI-DOTA-Gd-based polyplexes created in HBS showed similar results for 1/5, 1/15 and 1/25 ratio and overall seem to have similar transfection efficiency to LPEI under tested conditions.

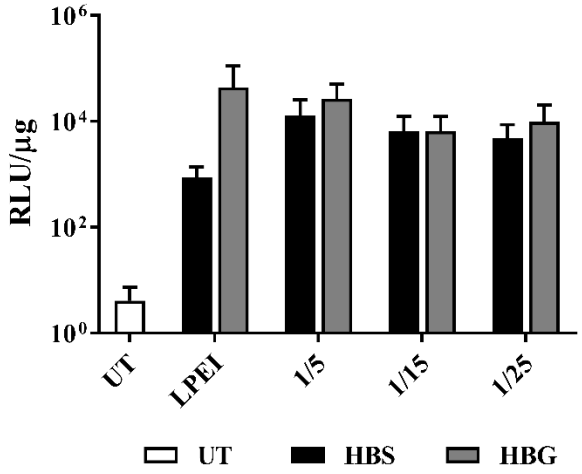

Figure 10. Transfection efficiency of LPEI-DOTA-Gd-based polyplexes compared to LPEI-based polyplexes tested on A549 cells.
A549 cells were treated 24 h with LPEI- and LPEI-DOTA-Gd (1/5, 1/15, 1/25)-based polyplexes generated at N/P 9 in 20 mM HEPES/150 mM NaCl (HBS) or in 20 mM HEPES/5 % (w/V) glucose (HBG) with pDNA encoding firefly luciferase gene (pCpG-hCMV-EF1α-LucSH) at final concentration 200 ng pDNA/well or were untreated (UT). 24 h after starting the treatment luciferase activity was measured. Data normalized on protein amount are shown (RLU/µg) as mean values of three independent experiments ± SD.

4.3 Nanoparticles mediated gene knockdown studies

4.3.1 Seeding density optimization for *in vitro* siRNA delivery studies

To optimize seeding density for siRNA delivery studies as well as to test the employment of BCA and Firefly luciferase based assay with MDA-MB-231-PGK-eGFP-Luc cells, both assays were read out 24 h after seeding different cell numbers/well (5,000 – 80,000 cells/well) as described in sections 3.6.1 and 3.7.

The total protein content for different cell numbers (Figure 11b), calculated based on standard curves (Figure 11a), acceptably increased in proportion to seeding number 10,000 and higher. However, the result for seeding number 5,000 was below signal threshold of BCA assay.

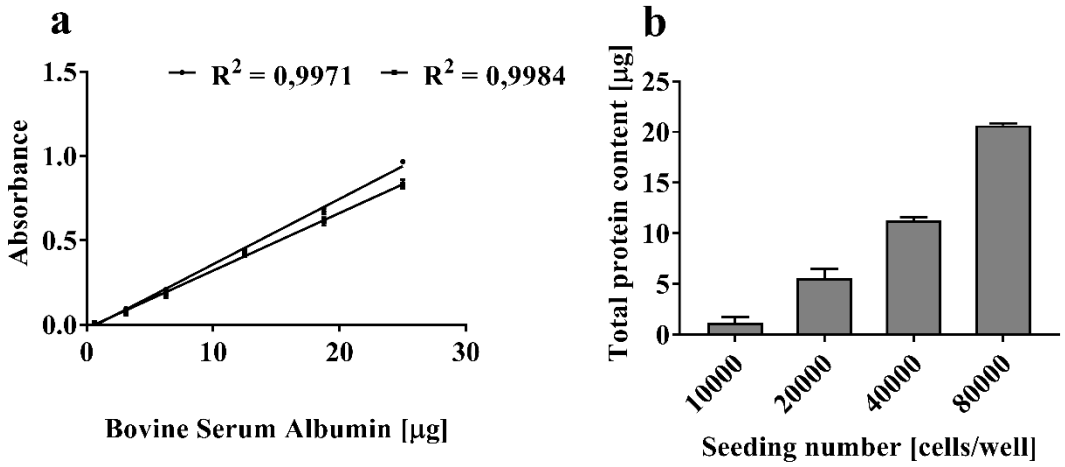

Figure 11. Seeding density optimization of MDA-MB-231-PGK-eGPF-Luc cells (Protein content).
10,000, 20,000, 40,000 and 80,000 MDA-MB-231-PGK-eGPF-Luc cells/well were seeded in white 96-well plate and incubated overnight. Then total protein content of wells (b) was determined by BCA assay and its calculation was based on bovine serum albumin standard curve (a) measured by the BCA assay for each independent experiment. Mean values of triplicates ± SD are shown (a). Mean values of two independent experiments ± SD are shown (b).

Results of Firefly luciferase based assay showed luminescence proportionally growing with seeding number ($R^2 = 0.9986$). As can be seen in Figure 12, RLU values higher than 10^6 including seeding number 5000 which makes employment of Firefly luciferase based assay using prepared Luciferase Assay Buffer suitable for MDA-MB-231-PGK-eGFP-Luc cells since the testing Luciferase Assay Buffer with recombinant luciferase showed reliable results for RLU values higher than 10^4(as described in section 4.1).

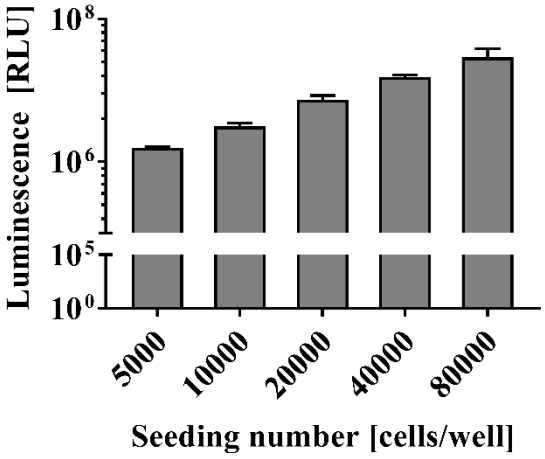

Figure 12. Seeding density optimization of MDA-MB-231-PGK-eGPF-Luc cells (Luminescence).
5,000, 10,000, 20,000, 40,000 and 80,000 MDA-MB-231-PGK-eGPF-Luc cells/well were seeded in white 96-well plate and incubated overnight. Then the luminescence was measured performing Firefly luciferase based reporter gene assay. Mean values of two independent experiments ± SD are shown.

When luminescence values (RLUs) were normalized by their total protein content determined by BCA assay (RLU/µg) they were again increasing with seeding number (R^2 = 0.9974). Naturally, as protein determination was not possible for seeding number 5,000, no normalization by protein content can be done for this seeding number.

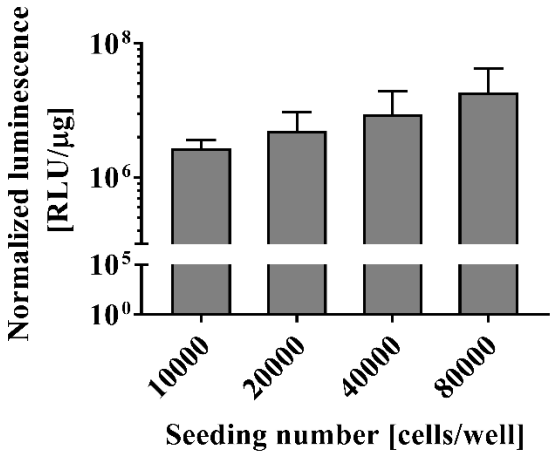

Figure 13. Seeding density optimization of MDA-MB-231-PGK-eGPF-Luc cells (Normalized luminescence).
10,000, 20,000, 40,000 and 80,000 MDA-MB-231-PGK-eGPF-Luc cells/well were seeded in white 96-well plate and incubated overnight. Then the luminescence was measured performing Firefly luciferase based reporter gene assay and the total protein content was measured by BCA assay (Figure 11). Data are shown as luminescence values (relative light units = RLU) normalized on protein content (RLU/µg). Mean values of two independent experiments ± SD are shown.

Based on above results, for seeding numbers around 20,000 – 40,000 both assays showed sufficient sensitivity. Considering the need of 48 h incubation in siRNA delivery *in vitro* studies (described in section 3.6), higher seeding numbers were not chosen. Thus, 20,000 and 30,000 seeding numbers were chosen for comparison by confluency assessment which is shown in Figure 14. According to Taschauer et al. (2016), optimal confluency for transfection by polyplexes is 70-80 %. As 20,000 and 30,000 cells/well did not show apparent difference in confluency, 30,000 cells/well was chosen as seeding density of MDA-MB-231-PGK-eGFP-Luc cells for later experiments with nanoparticles mediated siRNA delivery.

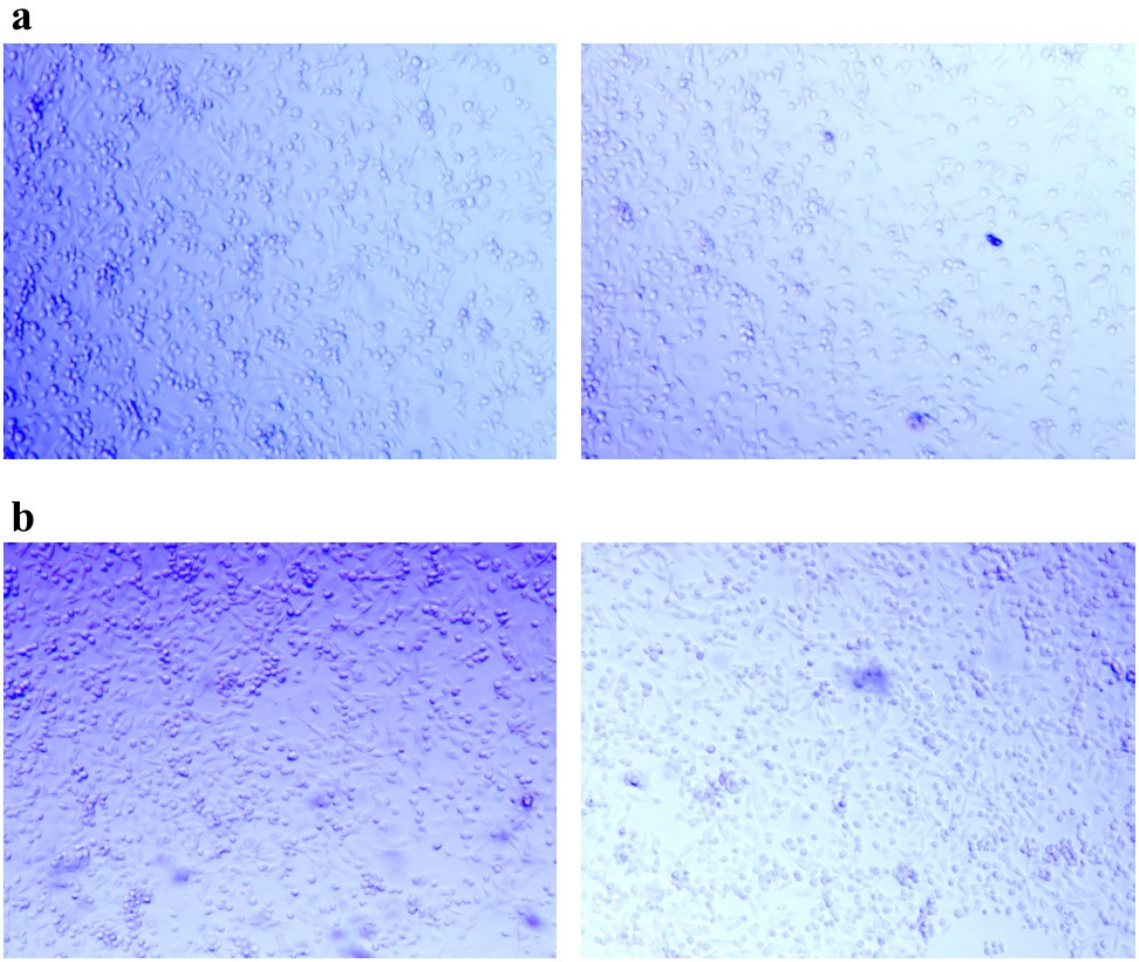

Figure 14. Confluency comparison of different cell seeding numbers by microscopy.
MDA-MB-231-PGK-eGPF cells were seeded at density 20,000 (a) or 30,000 (b) cells per well and incubated overnight, then the cell confluency was documented by Inverted Laboratory Microscope with LED Illumination (DM IL LED, Leica Microsystems, Wetzlar, Germany) equipped by Camera (DFC450, Leica Microsystems, Wetzlar, Germany).

4.3.2 LPEI/siRNA polyplexes

MDA-MB-231-PGK-eGFP-Luc cells were incubated with LPEI/siRNA polyplexes generated at N/P ratios 6, 9 and 20 to test for their siRNA delivery efficiency at final amount of siRNA 5 pmol/well. In initial experiment, cells (20,000 and 30,000 cells/well) were incubated for 48 h in complete media (48hC(I) incubation in Table 3 in section 3.6.2) with polyplexes generated in HBG with adding immediately to cells (t = 0 min). Results in Figure 15 show that no knockdown was achieved by all tested N/P ratios with no difference between 20,000 and 30,000 seeding numbers.

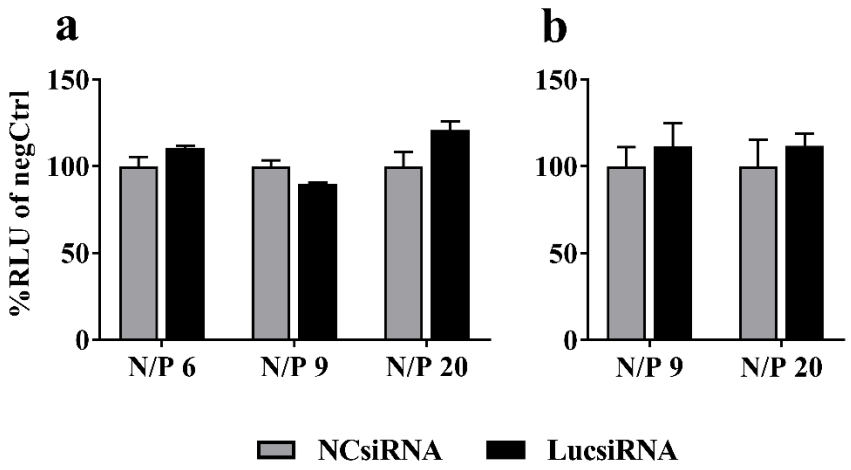

Figure 15. LPEI/siRNA polyplexes generated in HBG (5 pmol siRNA/well).
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 20,000 (a), 30,000 (b)) were incubated for 48 h in complete media with LPEI/siRNA polyplexes with final amount of siRNA 5 pmol/well. Polyplexes were generated in 20 mM HEPES/5 % (w/V) glucose (HBG) with t = 0 min after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Further, LPEI/siRNA polyplexes generated in HBS with or without incubation after polyplexing (t = 0 min or t = 30 min) were tested for knockdown.

Cells were incubated for 4 h in complete media (4hC incubation in Table 3 in section 3.6.2) with polyplexes generated in HBS with t = 30 min incubation after polyplexing. 48 h after starting the treatment luciferase activity was measured. Results presented in Figure 16 show that under tested conditions none of tested formulations achieved knockdown of the luciferase gene.

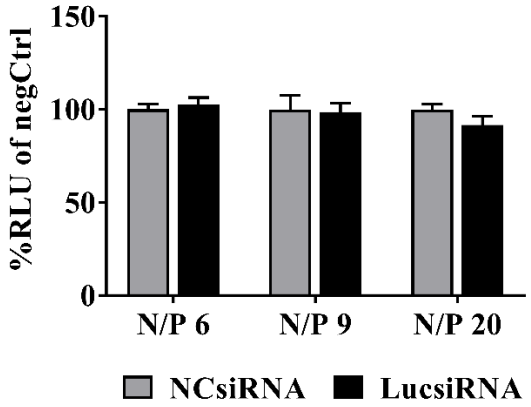

Figure 16. LPEI/siRNA polyplexes generated in HBS (5 pmol siRNA/well).
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 30,000) were incubated for 4 h in complete media with LPEI/siRNA polyplexes with final amount of siRNA 5 pmol/well. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with t = 30 min after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

To test another incubation condition and compare influence of incubation time after polyplexing, cells were incubated for 4 h in basal media (4hB(I) incubation in Table 3 in section 3.6.2) with polyplexes generated in HBS with or without incubation after polyplexing (t = 0 min or t = 30 min). As seen in Figure 17 no knockdown of luciferase gene was achieved by all tested formulations with no difference between t = 0 min and t = 30 min.

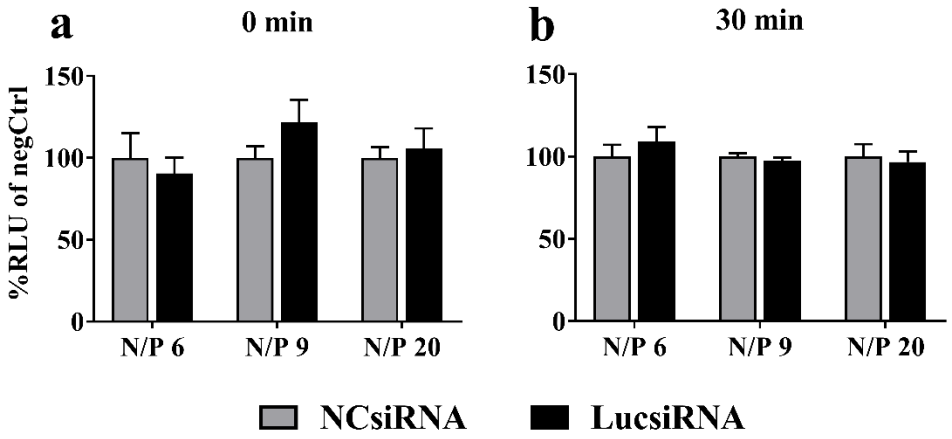

Figure 17. LPEI/siRNA polyplexes generated in HBS (5 pmol siRNA/well) II.
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 30,000) were incubated for 4 h in basal media with LPEI/siRNA polyplexes with final amount of siRNA 5 pmol/well. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with t = 0 or 30 min after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Overall, in experiments with LPEI/siRNA polyplexes with final amount of siRNA 5 pmol/well no knockdown of luciferase gene was achieved under tested conditions. Namely, among polyplex parameters N/P ratios 6, 9 and 20 and generation of polyplexes in HBS and HBG with or without incubation after polyplexing (t = 0 or 30 min) were tested. Among incubation parameters, 4 h in basal or complete (+ FBS, L-Glu, P/S) and 48 h in complete (+ FBS, L-Glu, P/S) media were tested.

4.3.3 BPEI/siRNA polyplexes

BPEI/siRNA polyplexes mediated gene knockdown studies using HeLa-PGK-eGFP-Luc cells

The gene silencing efficiency of BPEI/siRNA polyplexes (generated in HBS) were tested in HeLa-PGK-eGFP-Luc cells. No incubation time after polyplexing was applied (t = 0 min). Cells were treated with different concentrations of polyplexes, expressed as amount of siRNA/well, for 48 h in complete media but without antibiotics (48hC(II) incubation in Table 3 in section 3.6.2).

At 1 pmol siRNA/well, no reduction in luciferase expression was achieved in cells treated with polyplexes of N/P ratios 6 and 9. Only negligible reduction in luciferase expression was observed in cells treated with polyplexes of N/P ratio 20 as shown in Figure 18.

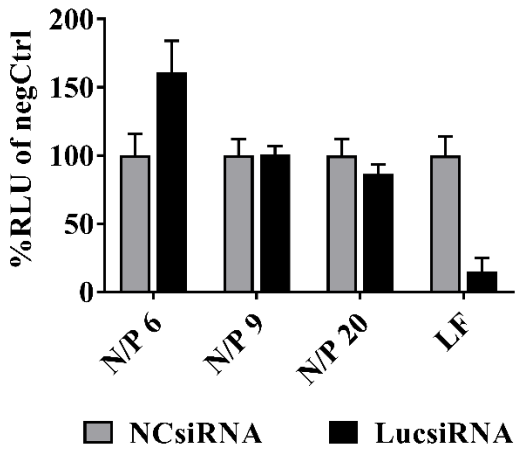

Figure 18. BPEI/siRNA polyplexes tested on HeLa-PGK-eGPF-Luc cells (1 pmol siRNA/well).
HeLa-PGK-eGPF-Luc cells were incubated for 48 h in complete media (without antibiotics) with BPEI/siRNA polyplexes at final amount of siRNA 1 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with t = 0 min after polyplexing. 48 h after starting the treatment the luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

For experiments with higher amounts of siRNA (5 pmol and 10 pmol siRNA/well) results are shown in Figure 19 using data analysis method 2, as described in section 3.8.1; since protein concentrations of some samples were not comparable with the concentration of negative control which makes the method 3 inappropriate. The possible reason for

the protein concentration differences can be problems with lysis of the HeLa-PGK-eGPF-Luc cells that occurred during the experiments. Especially, in experiment shown in Figure 19a, high error bars reflect the protein concentration deviations. Although a decrease in luciferase activity in cells treated with polyplexes at N/P ratio 9 and 20 (for both 5 and 10 pmol) is observed but error bars are very high (probably because of imperfect cell lysis). In experiment shown in Figure 19b, repetition for some samples was performed and wider range of N/P ratios was tested at 10 pmol siRNA/well. Although for 10 pmol the normalized luciferase activity (in percentage of UT) decreases with increase in N/P ratio, no gene silencing can be described since also negative control results decrease with the same tendency. However, also Lipofectamine® RNAiMAX based lipoplexes as positive control did not work in the experiment. Thus, no conclusion can be done from the experiment.

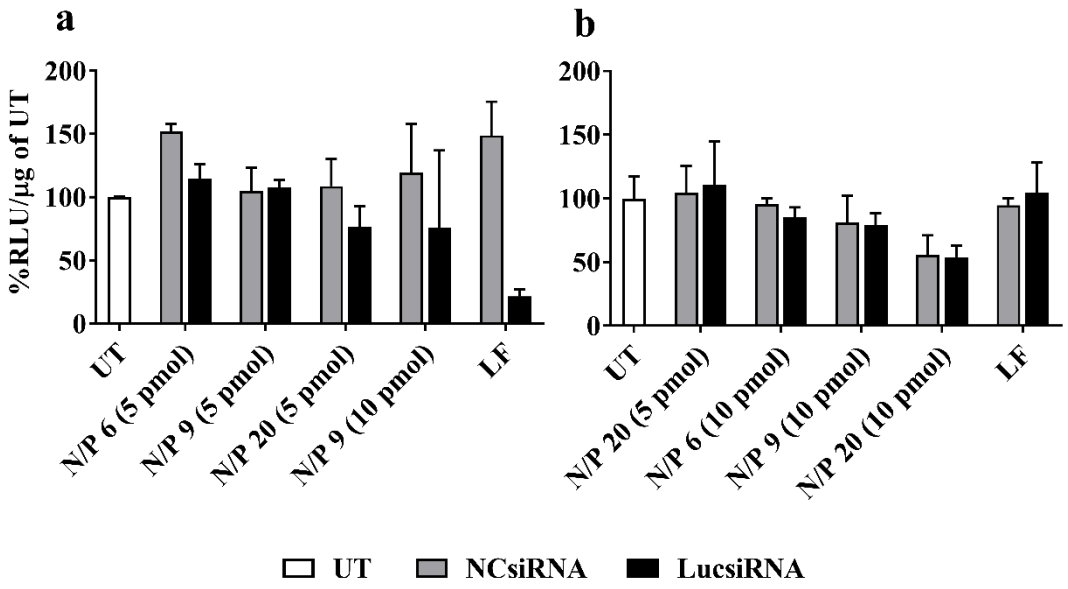

Figure 19. BPEI/siRNA polyplexes tested on HeLa-PGK-eGPF-Luc cells (5 and10 pmol siRNA/well).

HeLa-PGK-eGPF-Luc cells were incubated for 48 h in complete media (without antibiotics) with BPEI/siRNA polyplexes at final amount of siRNA 5 pmol/well and 10 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions or were untreated (UT). Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with $t = 0$ min after polyplexing. 48 h after starting the treatment the luciferase activity and total protein content were measured. Data are shown as %RLU/µg of untreated wells. Two independent experiments are shown (a and b). Mean values of triplicates ± SD are shown.

Because of probable insufficient lysis of HeLa-PGK-eGPF-Luc cells, as mentioned above, MDA-MB-231-PGK-eGFP-Luc cells were chosen as more appropriate model for gene silencing studies since no such problems were observed with this cell line.

BPEI/siRNA polyplexes mediated gene knockdown studies using MDA-MB-231-PGK-eGFP-Luc cells

The gene silencing efficiency of BPEI/siRNA polyplexes tested in MDA-MB-231-PGK-eGFP-Luc cells was determined for wide range of concentrations, incubation and polyplex parameters as described in section 3.6.2.

At final amount of siRNA 1 pmol/well polyplexes generated in HBS were tested at following parameters. Firstly, cells were incubated with polyplexes of N/P ratios 3, 6 and 9 for 4 h in basal media (4hB(I) incubation in Table 3 in section 3.6.2). No incubation time after generation of polyplexes was applied (t = 0 min). As can be seen in Figure 20, no decrease of luciferase activity was achieved by polyplexes tested.

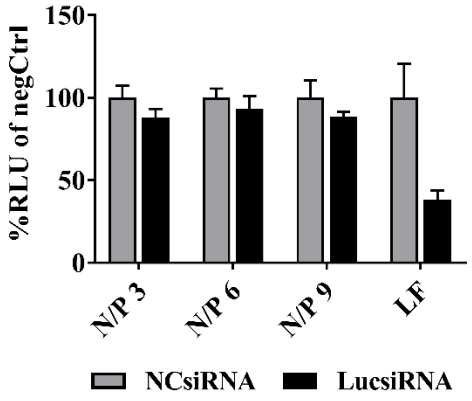

Figure 20. BPEI/siRNA polyplexes (1 pmol siRNA/well) - 4 h incubation in basal media. MDA-MB-231-PGK-eGFP-Luc cells were incubated for 4 h in basal media with BPEI/siRNA polyplexes at final amount of siRNA 1 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with t = 0 after polyplexing. 48 h after starting the treatment the luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Secondly, 48 h incubation was tested with comparison of incubation in complete media with antibiotics (+ FBS, + L-Glu, + P/S) and without antibiotics (+ FBS, + L-Glu), i.e. 48hC(I) and 48hC(II) incubation as described in Table 3 in section 3.6.2. Polyplexes

tested were generated with no incubation time after polyplexing (t = 0 min) and N/P ratios 3, 6, 9 and also 20 were tested. Results shown in Figure 21 indicate that no knockdown was achieved by polyplexes tested at both incubation conditions except from N/P 6 polyplexes which shown after 48 h incubation in complete media with antibiotics around 80 % luciferase activity of negative control. Result with N/P 6 polyplexes was not confirmed at other incubation conditions.

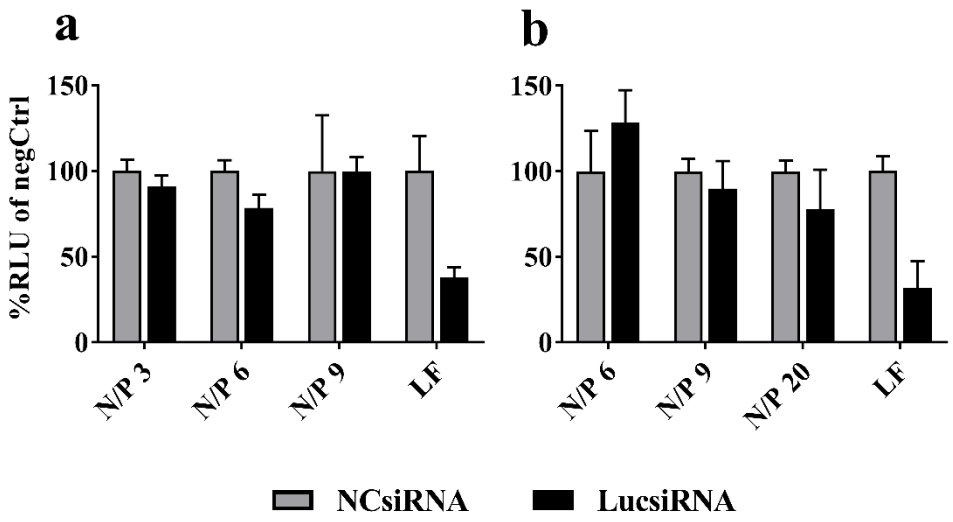

Figure 21. BPEI/siRNA polyplexes (1 pmol siRNA/well) - 48 h incubation in complete media.
MDA-MB-231-PGK-eGFP-Luc cells were incubated for 48 h in complete media containing antibiotics (a) or without antibiotics (b) with BPEI/siRNA polyplexes at final amount of siRNA 1 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with t = 0 min after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Lastly, polyplexes of N/P ratios 9 and 20 were incubated with cells for 48 h with starting the incubation in basal media for 4 h (4hB+44hC incubation in Table 3 in section 3.6.2). Polyplexes with t = 40 min incubation time after polyplexing were compared to those with t = 0 min. As shown in Figure 22, no decrease in luciferase activity was achieved by polyplexes tested. Also, no benefit resulted from incubation time t = 40 min after polyplexing for those polyplexes tested.

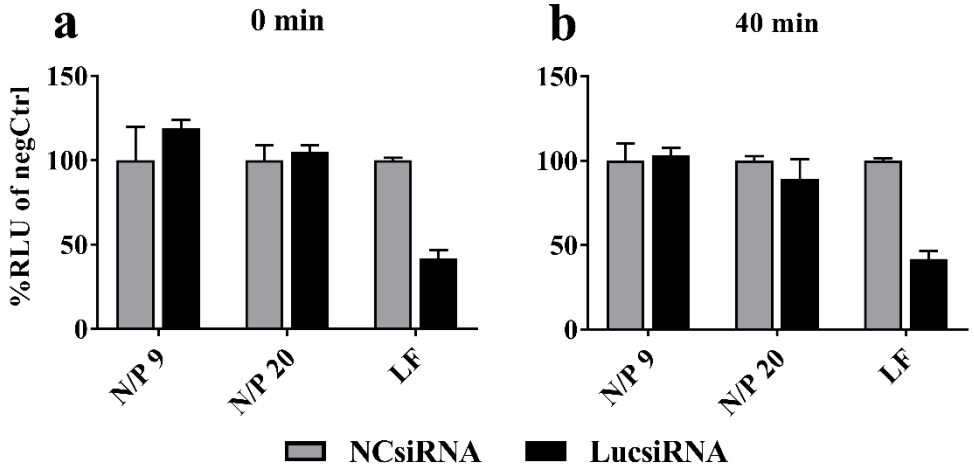

Figure 22. BPEI/siRNA polyplexes (1 pmol siRNA/well) – 48 h incubation starting in basal media.
MDA-MB-231-PGK-eGFP-Luc cells were incubated for 48 h with BPEI/siRNA polyplexes at final amount of siRNA 1 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions. The incubation with polyplexes was started in basal media and 4 h complete media without antibiotics was added. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with $t = 0$ min (a) or $t = 40$ min (b) after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Overall 1 pmol of siRNA/well appears to be insufficient for BPEI/siRNA polyplexes mediated gene silencing as no apparent knockdown was achieved under variation of incubation and polyplex parameters tested.

Also at final amount increased to 5 pmol and 10 pmol siRNA/well (Figure 23), polyplexes generated in HBS with no incubation time after polyplexing ($t = 0$ min) did not result in decrease of luciferase activity of MDA-MBA-231-PGK-eGFP-Luc cells treated with the polyplexes for 48 h in complete media without antibiotics, i.e. 48hC(II) incubation described in Table 3 in section 3.6.2.

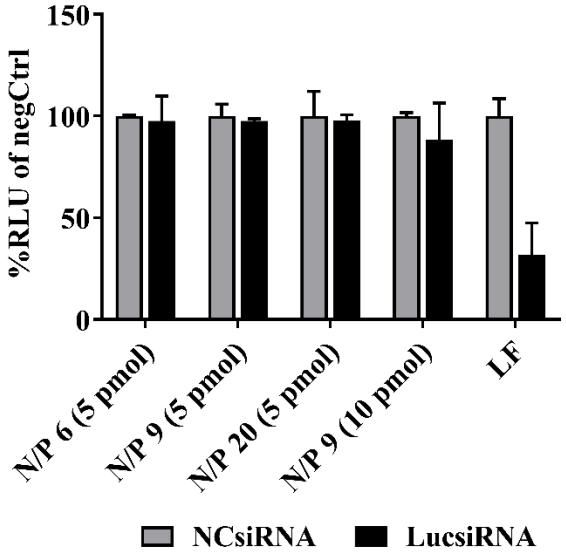

Figure 23. BPEI/siRNA polyplexes (5 and 10 pmol siRNA/well).
MDA-MB-231-PGK-eGFP-Luc cells were incubated for 48 h in complete media without antibiotics with BPEI/siRNA polyplexes generated in 20 mM HEPES/150 mM NaCl at final amount of siRNA 5 pmol/well and 10 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with $t = 0$ min after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of wells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Since no knockdown was achieved at amounts 1 – 10 pmol siRNA/well, cells were incubated with polyplexes of N/P ratio 9 at final amount 36 pmol siRNA/well. Taking into consideration a possible toxicity of high concentration of polyplexes both 4 h incubation (4hB(II) incubation in Table 3 in section 3.6.2) as well as 48 h incubation (4hB+44hC incubation in Table 3 in section 3.6.2) were tested. Figure 24(b, c) shows that polyplexes tested at 36 pmol siRNA/well achieved knockdown comparable as or higher than positive control Lipofectamine® RNAiMAX based lipoplexes. For polyplexes created in either in HBG or HBS, comparable efficiency can be seen with or without incubation time after polyplexing ($t = 0$ min or $t = 40$ min). Figure 24(b, c) indicates no profit from incubation after polyplexing. Comparison of polyplexes created in HBS and HBG is shown in Figure 24b and Figure 24c with comparable efficiency. Incubation conditions tested can be compared for polyplexes created in HBS with $t = 40$ min in Figure 24a and Figure 24b where 48 h incubation shows markedly better results in terms of knockdown than 4 h incubation and can be considered as appropriate incubation condition taking into account starting the incubation in basal media together with treating the cells for 48 h. Based on total protein content, judgement on toxicity of these

polyplexes can be done. Polyplexes created in HBG (Figure 24f) showed around 75 % of protein content of untreated samples, polyplexes in HBS showed decrease of protein content up to around 60 % of protein content of untreated samples with lower decrease for 4 h treatment (Figure 24d) and more noticeable decrease for 48 h treatment (Figure 24e).

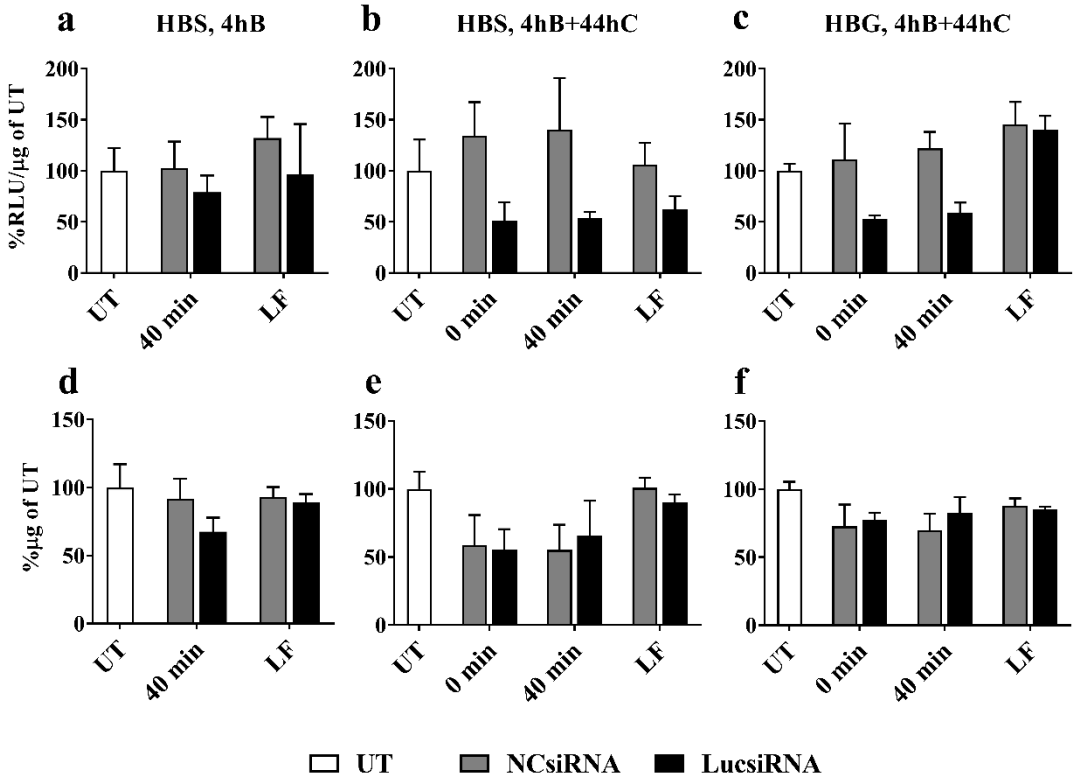

Figure 24. BPEI/siRNA polyplexes (36 pmol siRNA/well).
MDA-MB-231-PGK-eGFP-Luc cells were incubated for 4 h in basal media (4hB) (a, d) or for 48 h with starting the incubation in basal media and after 4 h adding complete media without antibiotics (4hB+44hC) (b, c, e, f) with BPEI/siRNA polyplexes at final amount of siRNA 36 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions or were untreated (UT). Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) (a, b, d, e) or in 20 mM HEPES/5 % (w/V) glucose (HBG) (c, f) with t = 0 or 40 min after polyplexing. 48 h after starting the treatment luciferase activity and total protein content were measured. Data are shown as normalized luciferase activity in %RLU/µg of untreated samples (a, b, c) or total protein content in %µg of untreated samples (d, e, f). Mean values of two independent experiments ± SD are shown.

4.3.4 c-LPEI/siRNA polyplexes

To test c-LPEI/siRNA polyplexes for knockdown, final amount 5 pmol and 10 pmol siRNA/well was used in initial experiment. Polyplexes were incubated with HeLa-PGK-eGFP-Luc cells for 48 h in complete media (+FBS, L-Glu) but without antibiotics (48hC(III) incubation in Table 3 in section 3.6.2). Polyplexes were generated in HBS with no incubation after polyplexing (t = 0 min). As seen in Figure 25 no knockdown was achieved under tested conditions by c-LPEI/siRNA polyplexes and only slight knockdown by Lipofectamine® RNAiMAX. Therefore, also possibly non-functional batch of siRNA should be considered as possible reason for this result.

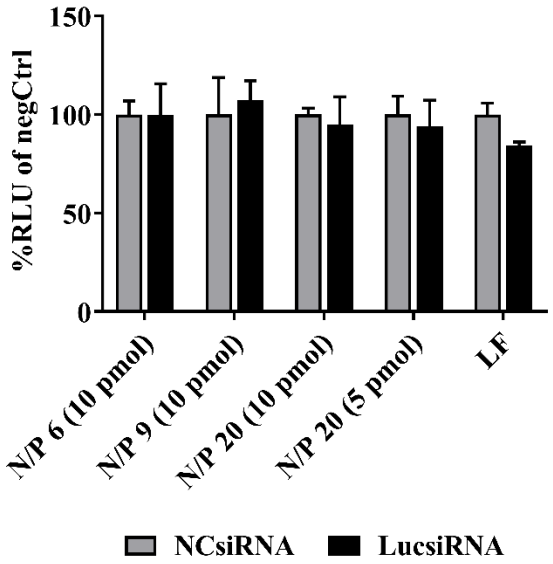

Figure 25. c-LPEI/siRNA polyplexes tested on HeLa-PGK-eGFP-Luc cells (5 and 10 pmol siRNA/well).
HeLa-PGK-eGFP-Luc cells were incubated for 48 h in complete media without antibiotics with c-LPEI/siRNA polyplexes at final amount of siRNA 5 pmol/well and 10 pmol siRNA/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions. Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) with t = 0 after polyplexing. 48 h after starting the treatment luciferase activity was measured. Data are shown as %RLU of cells treated with negative control polyplexes. Mean values of triplicates ± SD are shown.

Further, c-LPEI/siRNA polyplexes at same final amount (36 pmol siRNA/well), N/P ratio (N/P 9) and other parameters as BPEI/siRNA polyplexes that achieved knockdown (Figure 24) were incubated with MDA-MB-231-PGK-eGFP-Luc cell for 48 h starting with 4 h incubation in basal media with adding complete media (+FBS, L-Glu) but

without antibiotics, i.e. 4hB+44hC incubation described in Table 3 in section 3.6.2. Polyplexes generated in HBS or HBG with or without incubation after polyplexing (t = 0 or 40 min) were compared. As seen in Figure 26a, polyplexes generated in HBS with t = 0 min after polyplexing achieved the most apparent knockdown. The result for polyplexes generated in HBG with t = 40 min after polyplexing also indicates slight knockdown but the other types of polyplexes did not show efficient knockdown. However, the fact that also positive control (Lipofectamine® RNAiMAX based lipoplexes) did not result in gene silencing should be considered. Figure 26b shows total protein content of cells treated with these polyplexes was around 50-70 % of untreated samples.

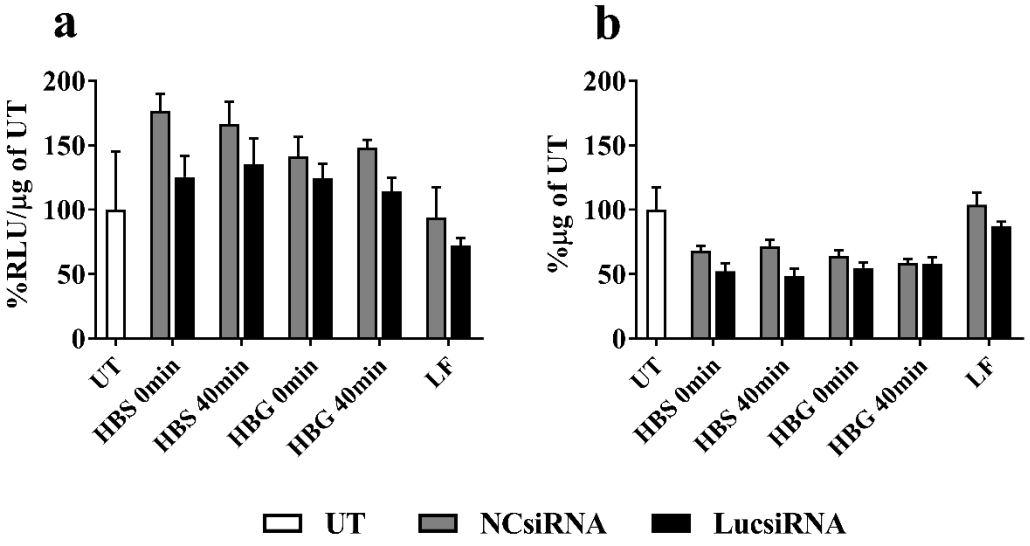

Figure 26. c-LPEI/siRNA polyplexes (36 pmol siRNA/well).
MDA-MB-231-PGK-eGFP-Luc cells were incubated for 48 h (with starting the incubation in basal media and after 4 h adding complete media without antibiotics) with BPEI/siRNA polyplexes at final amount of siRNA 36 pmol/well or were treated with Lipofectamine® RNAiMAX/siRNA lipoplexes (LF) according to manufacturer´s instructions or were untreated (UT). Polyplexes were generated in 20 mM HEPES/150 mM NaCl (HBS) or in 20 mM HEPES/5 % (w/V) glucose (HBG) with t = 0 or 40 min after polyplexing. 48 h after starting the treatment luciferase activity and total protein content were measured. Data are shown as normalized luciferase activity in %RLU/µg of untreated samples (a) and as total protein content in %µg of untreated samples (b). Mean values of triplicates ± SD are shown.

4.3.5 Layer-by-Layer assembled gold nanoparticles

Layer-by-Layer assembled gold nanoparticles (as described in section 3.4.2) are composed of different layers: gold nanoparticle (AuNP) represents a core, first layer is PEI, second layer siRNA and as third terminal layer is PEI again. Three different formulations containing BPEI or c-LPEI were tested on MDA-MB-231-PGK-eGFP-Luc:

A. AuNP-BPEI-siRNA-BPEI
B. AuNP-BPEI-siRNA-c-LPEI
C. AuNP-c-LPEI-siRNA-c-LPEI

These formulations were synthesized for both LucsiRNA (siRNA targeted against Firefly luciferase gene) and negative control NCsiRNA (siRNA with scrambled sequence which will not knockdown the luciferase gene) and were then used for testing gene knockdown efficiency.

A. Au-BPEI-siRNA-BPEI

The goal of the first experiment was to test for potential toxicity and to examine the whole transfection procedure (as described in 3.6.3) evaluated by BCA and Firefly luciferase based assay.

Formulations tested were AuNP-BPEI-Alexa750NCsiRNA-BPEI (NCsiRNA 1) and AuNP-BPEI-NCsiRNA-BPEI (NCsiRNA 2), both carrying only negative control siRNA of two types, and were added to MDA-MB-231-PGK-eGFP-Luc cells (seeded 20,000 cells/well) in five concentrations (I = the lowest, V = the highest) as can be seen in Table 6. The cells were treated with the nanoparticles for 48 h in complete media (+ FBS, + L Glu, + P/S), afterwards total protein content and luciferase activity was determined, as described in section 3.7., with the result shown in Figure 27. Although total protein content (Figure 27a) show decrease for the lowest concentration tested (I), the higher concentrations did not show any apparent decrease for AuNP-BPEI-NCsiRNA-BPEI and only slight decrease for AuNP-BPEI-Alexa750NCsiRNA-BPEI. Regarding the luciferase activity (Figure 27b), no decrease was caused by both formulations tested. Therefore, for the next experiments testing the formulations for gene knockdown efficiency, range of concentrations II-V, i.e. 10^5-10^7 particles/well, was chosen.

Table 6. Initial concentrations of Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI.

Formulation	AuNP-BPEI-Alexa750NCsiRNA-BPEI		AuNP-BPEI-NCsiRNA-BPEI	
Abbreviation	NCsiRNA 1		NCsiRNA 2	
Concentration	Particles/well	SD	Particles/well	SD
I	6,23E+04	2,75E+03	5,99E+04	2,08E+03
II	3,12E+05	1,37E+04	2,99E+05	1,04E+04
III	1,56E+06	6,87E+04	1,50E+06	5,21E+04
IV	7,79E+06	3,44E+05	7,48E+06	2,60E+05
V	3,90E+07	1,72E+06	3,74E+07	1,30E+06

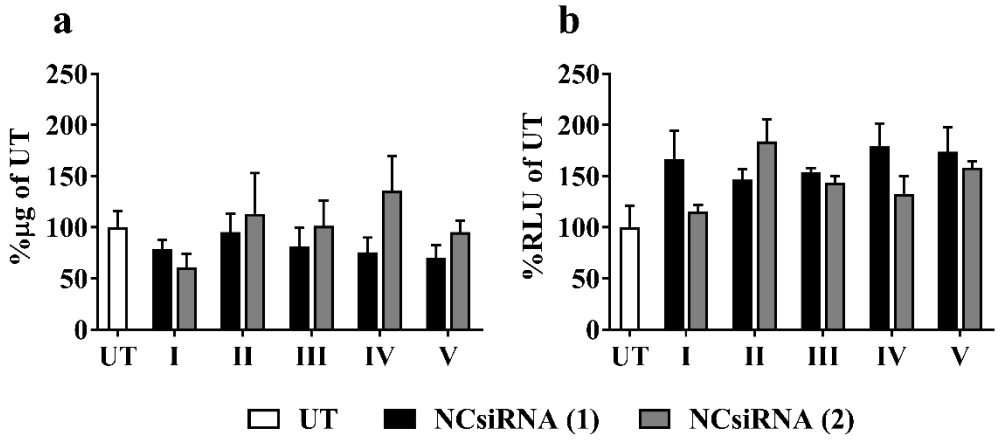

Figure 27. Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI. Effect on total protein content (a) and luciferase activity (b).
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 20,000 cells/well) were treated for 48 h in complete media (+ FBS, + L-Glu, + P/S) with AuNP-BPEI-Alexa750NCsiRNA-BPEI (NCsiRNA 1) or AuNP-BPEI-NCsiRNA-BPEI (NCsiRNA 2) nanoparticles at concentrations I(the lowest)-V(the highest) specified in Table 6 or were untreated (UT). Then the luciferase activity and total protein content were measured. Data are shown as mean values of triplicates ± SD.

To test for gene silencing efficiency, firstly AuNP-BPEI-siRNA-BPEI were incubated with MDA-MB-231-PGK-eGFP-Luc cells (seeded 20,000 cells/well) in 4 different concentrations (c1 = the lowest, c4 = the highest), specified in Table 7, for 48 h in complete media (+ FBS, + L-Glu, + P/S). As can be seen in Figure 28b, none of the concentrations led to decrease of luciferase activity, i.e. none of them caused the knockdown of the targeted gene. From protein concentrations, shown in Figure 28a, it can be estimated the concentrations were also not toxic, only the decrease in protein

content of samples treated with AuNP-BPEI-LucsiRNA-BPEI at concentrations c2 and c4 can be considered, however the same formulation carrying NCsiRNA did not lead to any decrease.

Table 7. Concentrations of Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI (c1-c5).

Formulation	AuNP-BPEI-siRNA-BPEI
Abbreviation	LucsiRNA or NCsiRNA
Concentration	Particles/well
c1	4,13E+05
c2	2,06E+06
c3	1,03E+07
c4	5,16E+07
c5	1,39E+08 – 2,06E+08

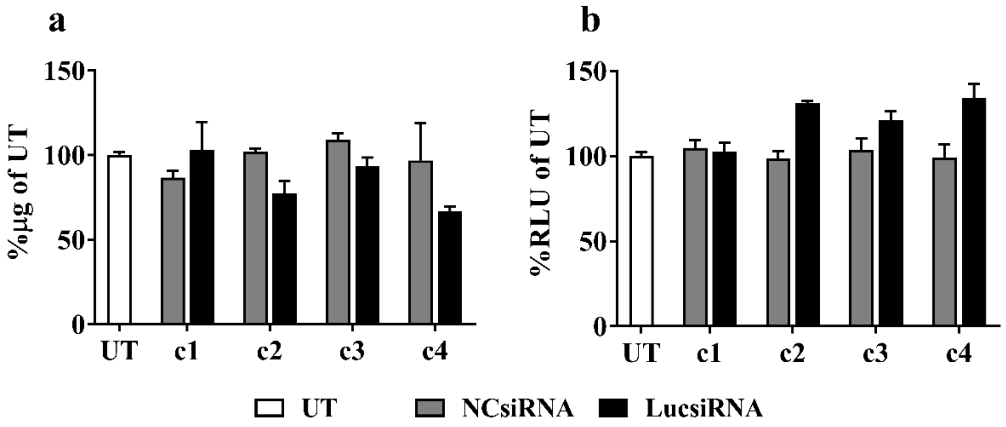

Figure 28. Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI (concentrations c1 – c4). Effect on total protein content (a) and gene silencing efficiency(b). MDA-MB-231-PGK-eGFP-Luc cells (seeding number 20,000 cells/well) were treated for 48 h in complete media (+ FBS, + L-Glu, + P/S) with AuNP-BPEI-siRNA-BPEI nanoparticles carrying either siRNA targeted against luciferase gene (LucsiRNA) or negative control siRNA (NCsiRNA) at concentrations c1(the lowest)-c4(the highest) specified in Table 7 or were untreated (UT). Then the luciferase activity and total protein content were measured Data are shown as mean values of triplicates ± SD.

Since no knockdown was achieved by the concentrations c1-c4, same experiment (with 20,000 cells seeded/well) was carried out with the nanoparticles at concentration c5 (Table 7) as well as with 30,000 cells seeded/well. From the results shown in Figure 29 it can be estimated that this concentration of nanoparticles led to a toxic effect on the cells

as the total protein content is markedly lower compared to untreated cells. Decrease of luciferase activity was caused not only by formulation carrying targeted siRNA but also by the formulation carrying negative control siRNA. However, slight knockdown of luciferase gene is indicated in Figure 29d, in which the incubation with 30,000 cells/well is shown, as the decrease of luciferase activity caused by formulation carrying LucsiRNA is greater.

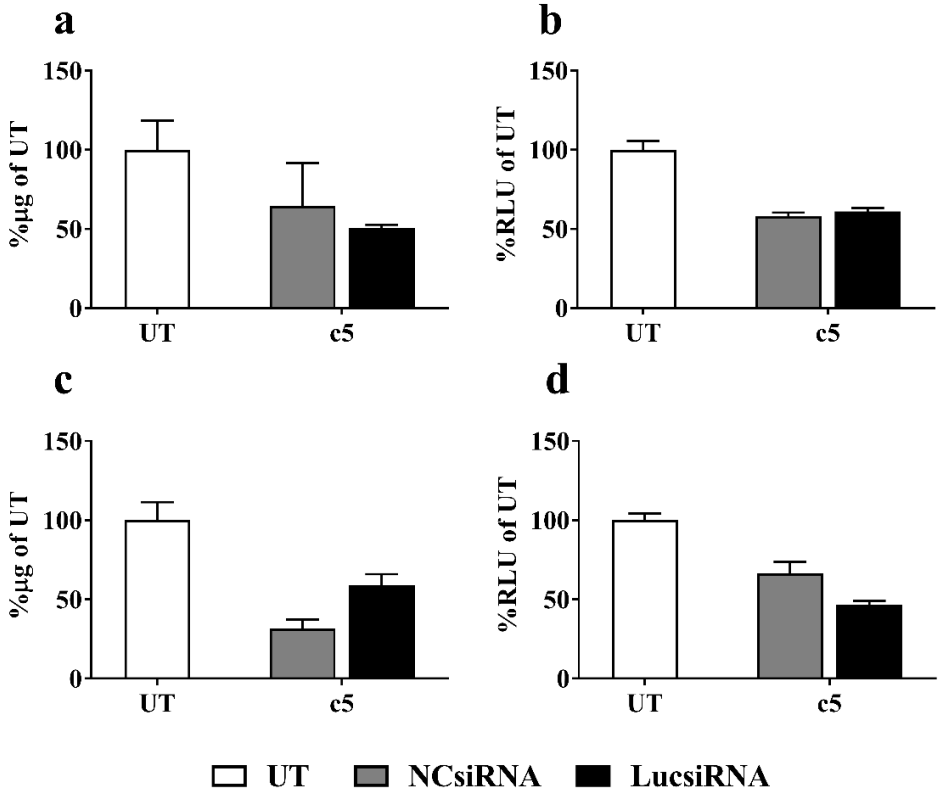

Figure 29. Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI (concentration c5). Effect on total protein content (a, c) and gene silencing efficiency(b, d). MDA-MB-231-PGK-eGFP-Luc cells (seeding number 20,000 cells/well (a,b) and seeding number 30,000 cells/well (c,d)) were treated for 48 h in complete media (+ FBS, + L-Glu, + P/S) with AuNP-BPEI-siRNA-BPEI nanoparticles carrying either siRNA targeted against luciferase gene (LucsiRNA) or negative control siRNA (NCsiRNA) at concentrations c5 (1,39E+08 – 2,06E+08 particles/well as shown in Table 7) or were untreated (UT). Then the luciferase activity and total protein content were measured. Data are shown as mean values of triplicates ± SD.

Further two higher concentrations c6 and c7, specified in Table 8, of AuNP-BPEI-siRNA-BPEI nanoparticles were tested with MDA-MB-231-PGK-eGFP-Luc cells. As can be seen in Figure 30, both concentrations were toxic which manifested itself as no measurable protein concentration and luciferase activity of samples after 48 h incubation.

Table 8. Concentrations of Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI (c6-c7).

Formulation	AuNP-BPEI-LucsiRNA-BPEI		AuNP-BPEI-NCsiRNA-BPEI	
Abbreviation	LucsiRNA		NCsiRNA	
Concentration	Particles/well	SD	Particles/well	SD
c6	8,94E+08	3,53E+07	1,23E+09	3,57E+07
c7	1,06E+09	7,07E+07	1,74E+09	6,19E+07

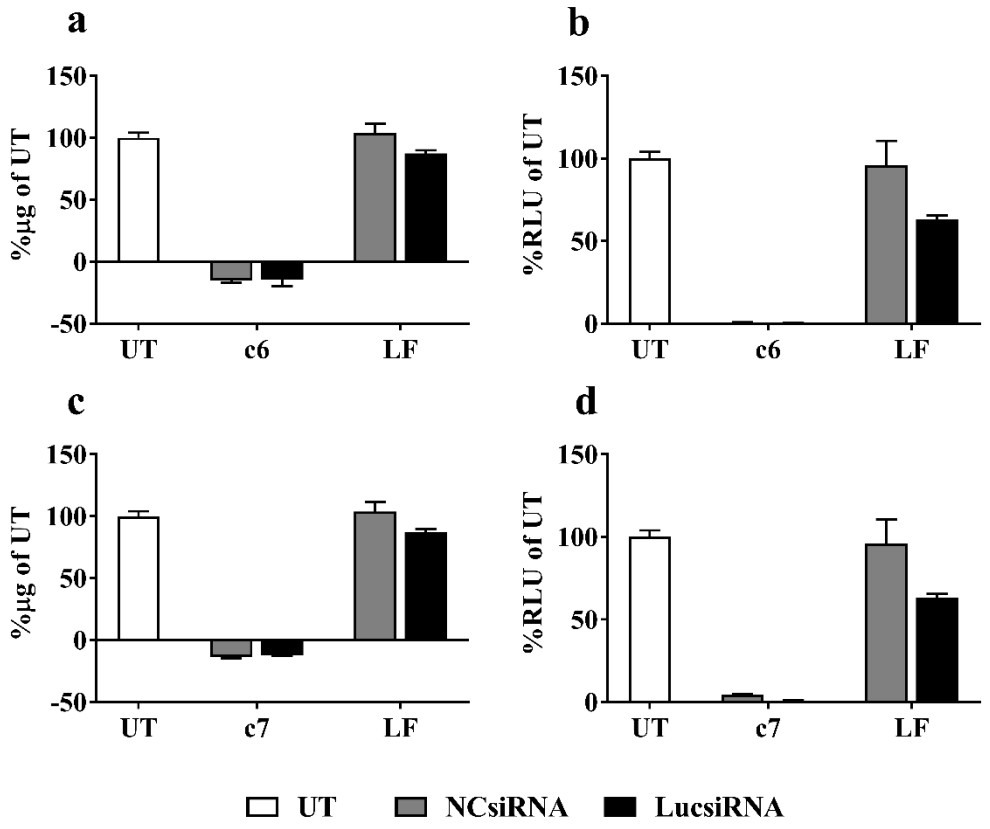

Figure 30. Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-BPEI (concentration c6 and c7). Effect on total protein content and gene silencing efficiency.
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 30,000 cells/well) were treated for 48 h in media (+ FBS, + L-Glu, without antibiotics) with AuNP-BPEI-siRNA-BPEI nanoparticles at concentrations c6 (a, b) or c7 (c, d), specified in Table 8, or with Lipofectamine® RNAiMAX based lipoplexes or were untreated (UT). Formulations were carrying either siRNA targeted against luciferase gene (LucsiRNA) or negative control siRNA (NCsiRNA). After the 48 h, the luciferase activity and total protein content were measured. Data are shown as mean values of triplicates ± SD.

B. Au-BPEI-siRNA-c-LPEI

To test for gene silencing efficiency, AuNP-BPEI-siRNA-c-LPEI were firstly incubated with MDA-MB-231-PGK-eGFP-Luc cells (seeded 20,000 cells/well) in 4 different concentrations (c1 = the lowest, c4 = the highest), specified in Table 9, for 48 h in complete media (+ FBS, + L-Glu, + P/S). As can be seen in Figure 31b, the formulation in concentrations tested was not efficient in the gene silencing. Also at these concentrations no toxicity was observed as the protein content shown in Figure 31a decreased slightly compared to untreated cells only in some concentrations for either formulation carrying LucsiRNA or NCsiRNA but never for both. Also, an unspecific toxicity would manifest also in decrease of luciferase activity which in this experiment remained unchanged (Figure 31b).

Table 9. Concentrations of Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-c-LPEI.

Formulation	AuNP-BPEI-siRNA-c-LPEI
Abbreviation	LucsiRNA or NCsiRNA
Concentration	Particles/well
c1	4,13E+05
c2	2,06E+06
c3	1,03E+07
c4	5,16E+07
c5	1,39E+08 – 2,06E+08

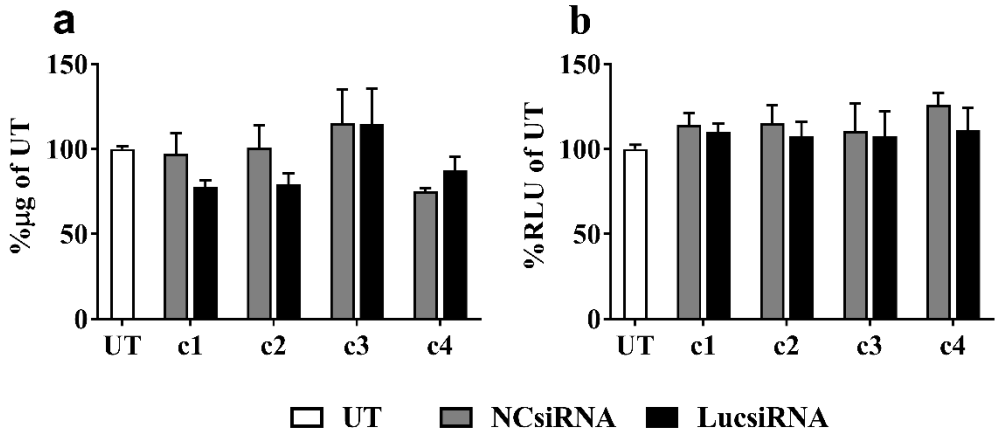

Figure 31. Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-c-LPEI (concentrations c1 – c4). Effect on total protein content (a) and gene silencing efficiency(b). MDA-MB-231-PGK-eGFP-Luc cells (seeding number 20,000 cells/well) were treated for 48 h in complete media (+ FBS, + L-Glu, + P/S) with AuNP-BPEI-siRNA-c-LPEI nanoparticles carrying either siRNA targeted against luciferase gene (LucsiRNA) or negative control siRNA (NCsiRNA) at concentrations c1(the lowest)-c4(the highest) specified in Table 9 or were untreated (UT). Then the luciferase activity and total protein content were measured. Data are shown as mean values of triplicates ± SD.

Further, the experiment under same conditions was carried out with concentration c5 (specified in Table 9). As can be seen in Figure 32, concentration c5 of AuNP-BPEI-siRNA-c-LPEI caused toxic effect which is firstly shown by the extensive decrease in protein content of samples compared to untreated cells (Figure 32a). Although the decrease in luciferase activity (Figure 32b) caused by formulation carrying the targeted siRNA greater than the decrease caused by the negative control formulation might indicate an ability of gene silencing, such large decrease in luciferase activity caused by negative control formulation suggests the cause of the decrease is rather the toxicity than the knockdown.

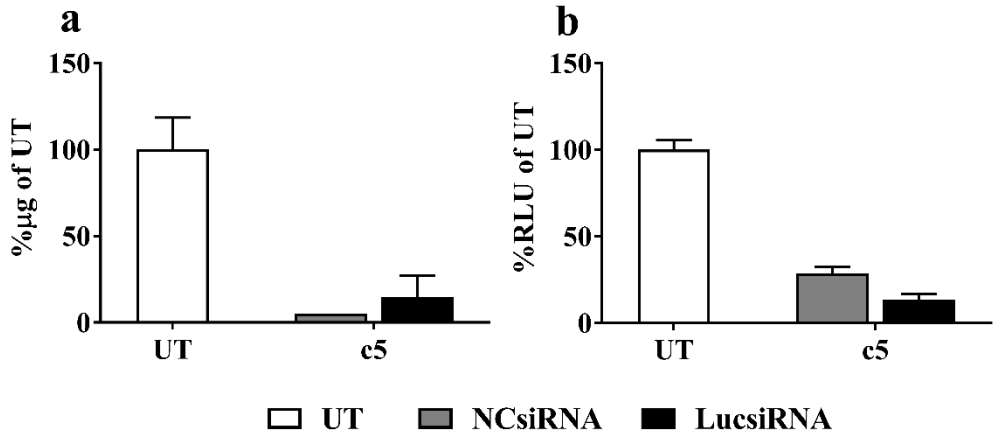

Figure 32. Layer-by-Layer assembled gold nanoparticles: AuNP-BPEI-siRNA-c-LPEI (concentration c5). Effect on total protein content (a) and gene silencing efficiency(b).
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 20,000 cells/well) were treated for 48 h in complete media (+ FBS, + L-Glu, + P/S) with AuNP-BPEI-siRNA-c-LPEI nanoparticles carrying either siRNA targeted against luciferase gene (LucsiRNA) or negative control siRNA (NCsiRNA) at concentration c5 (1,39E+08 – 2,06E+08 particles/well as shown in Table 9 or were untreated (UT). Then the luciferase activity and total protein content were measured. Data are shown as mean values of triplicates ± SD.

C. AuNP-c-LPEI-siRNA-c-LPEI

Formulation based on only cross-linked LPEI (AuNP-c-LPEI-siRNA-c-LPEI) was tested only at one concentration specified in Table 10. MDA-MB-231-PGK-eGPF-Luc cells were incubated with the nanoparticles for 48 h in complete media (+ FBS, + L-Glu) without antibiotics. Interestingly, the result (as seen in Figure 33**)** shows no apparent toxicity in case of concentration in range of 10^8, i.e. concentration c5-c6 which showed toxicity when applying AuNP-BPEI-siRNA-BPEI or AuNP-BPEI-siRNA-c-LPEI (c5) which can correlate with lower toxicity of c-LPEI compared to BPEI. However, as can be seen in Figure 33b no apparent gene knockdown was indicated by decrease of luciferase activity.

Table 10. Concentrations of Layer-by-Layer assembled gold nanoparticles: AuNP-c-LPEI-siRNA-c-LPEI.

Formulation	AuNP-c-LPEI-LucsiRNA-c-LPEI		AuNP-c-LPEI-NCsiRNA-c-LPEI	
Abbreviation	LucsiRNA		NCsiRNA	
Concentration	Particles/well	SD	Particles/well	SD
c(Luc) or c(NC)	4,61E+08	1,99E+07	1,05E+08	4,00E+06

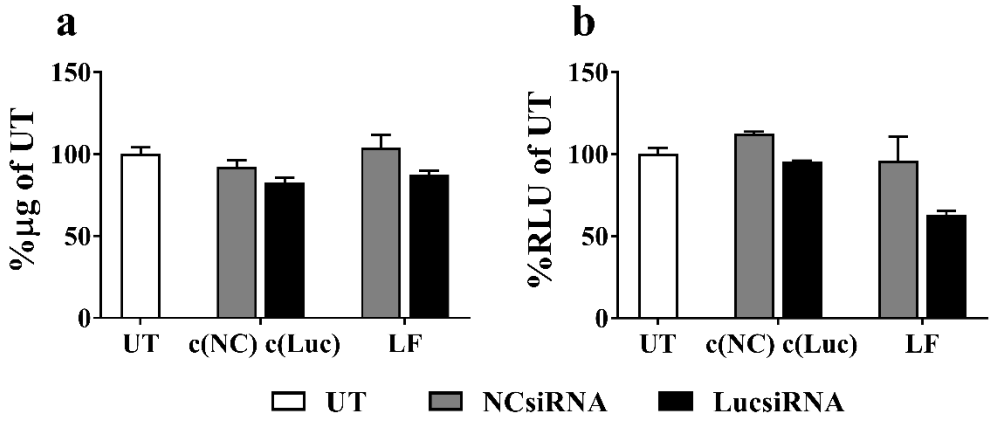

Figure 33. Layer-by-Layer assembled gold nanoparticles: AuNP-c-LPEI-siRNA-c-LPEI Effect on total protein content and gene silencing efficiency.
MDA-MB-231-PGK-eGFP-Luc cells (seeding number 30,000 cells/well) were treated for 48 h in complete media (+ FBS, + L-Glu, without antibiotics) with AuNP-c-LPEI-siRNA-c-LPEI nanoparticles at concentrations specified in Table 10 or with Lipofectamine® RNAiMAX based lipoplexes or were untreated (UT). Formulations were carrying either siRNA targeted against luciferase gene (LucsiRNA) or negative control siRNA (NCsiRNA). After the 48 h, the luciferase activity and total protein content were measured. Data are shown as mean values of triplicates ± SD.

5. DISCUSSION

The main aim of this study was evaluation of *in vitro* pDNA and siRNA delivery, mediated by different nanoparticles by Firefly luciferase reporter gene assay.

Delivery of pDNA was aimed at testing LPEI-DOTA-Gd, i.e. LPEI labelled with gadoteric acid newly synthetized for *in vivo* imaging purposes. To explore an effect of labelling the LPEI on its transfection ability, polyplexes composed of either LPEI or LPEI-DOTA-Gd and pDNA encoding Firefly luciferase gene (pCpG-hCMV-EF1α-LucSH) were tested on CT26 and A549 cells. From testing on CT26, it was observed that N/P 9 shows better transfection efficiency than N/P 6. The influence of N/P ratio on properties of polyplexes is also described in section 1.3, as N/P ratio influence mostly size and charge of polyplexes, in some cases also the presence of free polyethylenimine, all affecting the transfection efficiency (Scholz and Wagner 2012). However, when our results are compared with those of Taschauer et al. (2016) which includes an optimized protocol for *in vitro* transfection of A549 cells with LPEI-polyplexes of the same composition as in this work (LPEI 10 kDa, pCpG-hCMV-EF1α-LucSH, formed in HBG), we reached lower luciferase activities in general. As Taschauer et al. (2016) also state, *in vitro* transfection protocol should be optimized for each cell line. This was observed in case of CT26 cells where there was in-sufficient lysis, which is also a reason for showing the data as RLU values unnormalized on the total protein content. Therefore, a definitive comparison was done following the already optimized protocol with A549 cell line. Although results achieved by LPEI-based polyplexes were still lower than those presented by Taschauer et al. (2016), comparison of LPEI-DOTA-Gd and LPEI transfection ability was sufficiently displayed. As can be seen in Figure 10, transfection ability of LPEI-DOTA-Gd was comparable with or even slightly better than that of LPEI. On the other hand, it should be also considered that measured values are ranging around 10^4 which represents, based on measurements of sensitivity of the housemade Luciferase Assay Buffer (shown in section 4.1), an approximate limit of accuracy of the Firefly luciferase based assay.

Polyplexes mediated siRNA delivery studies aimed at exploring wide range of incubation and polyplex parameters resulting in finding the optimal parameters for efficient delivery. To find appropriate incubation conditions, incubation with different duration and in differently supplemented cell culture media was tested, namely in complete media

(supplemented with fetal bovine serum (FBS), penicillin and streptomycin (P/S) and L-Glutamine (L-Glu)), in media without antibiotics P/S and in basal media containing neither P/S nor FBS and L-Glu.

In recent siRNA delivery *in vitro* studies, 48 h is the most frequently used period between starting the transfection and assaying the gene knockdown efficiency (e.g. by assaying the luciferase activity), as described e.g. by Grayson et al. (2006), Tarcha et al. (2007), Zintchenko et al. (2008) and Hattori et al. (2014). Also in this work, the 48 h period was applied, incubating cells either without changing the media during the 48 h (48 h incubation) or with changing the media after 4 h post transfection (4 h incubation). The change of media after 4 h or after different time is also often used in the studies, e.g. by Breunig et al. (2008). The reason we included also this type of incubation was to provide the cells with a fresh medium free of any potentially toxic formulations and containing important nutrients and growth factors. Kichler et al. (2001) explains that within 4 h endocytosis of polyplexes is believed to be accomplished.

Especially, the change was necessary when starting the transfection in a basal media. The basal media is used due to absence of protein rich serum. Performing nanoparticles mediated siRNA delivery experiments in serum-free (e.g .Breunig et al. 2008) as well as in serum containing medium, mostly 10 % described e.g. by Zintchenko et al. (2008), is both quite often. An ability of serum to interact with transfection is a frequently discussed topic. Tros de Ilarduya and Düzgüneş (2000) described the ability of serum to inhibit the transfection mediated by a certain type of lipoplexes. Dai and Liu (2011) mention a non-specific adsorption of polycationic vectors on proteins which they explore in terms of *in vivo* application. When polyelectrolyte/DNA complexes are administered intravenously a colloidal aggregation comes about leading to rapid clearance from bloodstream. Taschauer et al. (2016) describe a formation of aggregates as a consequence of interaction of PEI polyplexes with blood components leading to transfection of lung tissue.

Though rarely, in some *in vitro* studies of nanoparticles mediated nucleic acid delivery, applying of cell culture medium containing FBS but no antibiotics has been shown. Koide et al. (2016) incubated B16F10-Luc2 cells with lipoplexes for siRNA delivery in medium supplemented with 10 % FBS but containing no antibiotics. The effect of antibiotics on transfection efficiency was explored by Jacobsen et al. (2004) using for pDNA delivery

in NIH/3T3 cells FuGENE 6 Transfection Reagent, a lipid-based transfection reagent (Roche Molecular Biochemicals 2001). Transfection levels dramatically decreased when combination of penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2,5 µg/ml) was present in cell culture media at time of transfection. However, as they mention, such parameter is usually cell line dependent. On the other hand, as explained by Thermo Fisher Scientific (2017c), medium for transient transfection can contain antibiotics. Further it is explained, that cationic lipid reagents increase cell permeability, thus cytotoxicity caused by increased amount of antibiotics delivered in cells can disturb transfection efficiency. However, this thesis included polyethylenimine-based vectors, for which no evidence of negative effect of antibiotics on transfection efficiency can be found. Nevertheless, presence and absence of antibiotics in medium was chosen as one of parameters for finding the optimal incubation conditions and based on this thesis data no significant difference was observed. However, as most of our experiments did not result in efficient knockdown, such conclusion should be confirmed by cfurther experiments with efficient delivery resulting in knockdown of luciferase gene. Such results were achieved in experiments with 36 pmol siRNA/well, however, those experiments were carried out without antibiotics.

Based on the reasons mentioned above and based on the proved efficient delivery under following conditions (Figure 24), 48 h incubation starting in basal media with adding complete media after 4 h was chosen as an appropriate incubation for PEI-based polyplexes mediated siRNA delivery in MDA-MB-231-PGK-eGFP-Luc cells. By starting transfection in basal media, possible interaction with serum proteins should be considerably avoided since the endocytosis is believed to be accomplished at that time point. Since a changing media as a critical step can also influence cells e.g. in their growth, not changing but adding the complete media to provide nutrients and growth factors was chosen. Comparison of those conditions can be seen in Figure 24 with the lowest transfection efficiency observed at 4 h incubation followed by changing of media.

Examined polyplex parameters included usage of different buffers for polyplex formation, applying incubation time after polyplex formation and different composition of polyplexes, namely ratio of polymer and siRNA expressed as N/P ratio, which stands for molar ratio of nitrogens from the PEI and phospates from nucleic acid, and type of PEI used. Though it was already published that branched polyethylenimine (BPEI) can

suit better for siRNA delivery than LPEI (Scholz and Wagner 2012, Kwok and Hart 2011), the aim of this work was to test and compare LPEI, BPEI and also disulfide crosslinked LPEI (c-LPEI), with promising biodegradable properties, for polyplexes mediated siRNA delivery.

Though many of parameters were tested when using 1 pmol, 5 pmol and 10 pmol siRNA per well, no knockdown was achieved independently on either incubation or polyplex parameters. Only when applying 36 pmol siRNA per well, the knockdown comparable to or better than positive control was achieved at some parameters. However, the final concentration of siRNA per well was 36 folds higher than the positive control, since 1 pmol siRNA per well was always applied by Lipofectamine® RNAiMAX based lipoplexes. Thus, final siRNA amount used can be considered as a superior parameter and was chosen, apart from type of PEI, as a leading parameter for organization of results of this work. Some results from previous studies with BPEI (25 kDa)-based polyplexes performed in 96-well plate format can be compared to our results. Grayson et al. (2006) tested a range 0,125 – 25 pmol siRNA per well on HR5-CL11 cells, a HeLa derivative, seeded in density 8000 cells/well. A significant knockdown was achieved only with 25 pmol siRNA/well. Oskuee et al. (2010) used in their study 38 pmol siRNA per well to test on Neuro2A-eGFPLuc cells seeded in density 5000 cells/well. However, using higher amounts of siRNA results in higher amount of PEI needed for complexation which is a limiting parameter because of PEI cellular toxicity, as also describe Taschauer et al. (2016). This is shown in the study by Oskuee et al. (2010) in which at 38 pmol siRNA per well in BPEI (25 kDa)-based polyplexes were unable of efficient delivery resulting in knockdown without apparent toxicity. In this thesis, interestingly, delivery by BPEI (25 kDa)-based polyplexes at similar amount of siRNA (36 pmol siRNA/well) led at specific parameters to efficient knockdown of the targeted luciferase gene. A judgement on toxicity can be done from carried out BCA assay, where the BPEI-based polyplexes showed around 60-85 % total protein content of untreated cells. Compared to Oskuee et al. (2010) we used considerably higher seeding density. Of course, differences between cell lines should also be considered. However, an interesting way of expressing final siRNA amount in recent studies is worth mentioning, e.g. Pinel et al. (2014) express siRNA amount in amol/cell.

Anyway, the correlation of polyethylenimine efficiency and toxicity associated particularly with molecular weight and polyethylenimine structure has been shown

(Breunig et al. 2007). Wightman et al. (2001) and Taschauer et al. (2016) state that branched versions of PEI are less biocompatible and more toxic than linear versions. In correlation with that, Breunig et al. (2008) who tested PEI/siRNA polyplexes using LPEI (5 kDa), c-LPEI (with disulfide bonds) and BPEI (25 kDa) showed that a relative cell viability was reduced at most by BPEI-, slightly less by c-LPEI- and was not influenced by LPEI-based polyplexes which correlates with the branching which was the highest for BPEI. In the same study, the polyplexes were also tested for cellular uptake and silencing efficiency. For both parameters, LPEI was the least effective. Uptake correlated again with branching in increasing order, i.e. BPEI-based polyplexes were taken up by the most of cells. Though c-LPEI-based polyplexes were taken up by lower number of cells, they showed a greater intracellular release of siRNA, most likely thanks to intracellular reduction of disulfide linkages. Regarding the silencing efficiency, reduction of expression of targeted gene was achieved in the following order: BPEI>c-LPEI>LPEI. However, use of BPEI caused also unspecific silencing effects resulting in conclusion that the c-LPEI was in the gene silencing more effective.

Results of this work, briefly, show that by the use of BPEI gene silencing effect was achieved, but with c-LPEI-based polyplexes at the same final siRNA amount (36 pmol/well) the gene silencing efficiency was markedly lower. Also, a decrease in protein content in comparison to untreated cells, which can correlate with toxicity, was slightly greater for samples treated with c-LPEI-based polyplexes than with BPEI-based polyplexes. When looking for reason why this result differs from those presented by Breunig et al. (2008) it should be considered that in this thesis only one experiment was carried out and thus the biological variability could cause such a difference. Also, the employment of positive control (Lipofectamine® RNAiMAX) in this case did not lead to the desirable results and, compared to Breunig et al. (2008) who tested wide range of N/P ratios (N/P 6 – N/P 48) with the efficiency achieved with N/P 12 and higher, only N/P ratio 9 was tested in our study. Regarding the final siRNA amount, they applied 24 pmol siRNA/well when using 24-well plate and seeding 38000 CHO-K1/EGFP cells/well.

Although LPEI-based polyplexes were also tested in this thesis, comparison with BPEI and c-LPEI would require performing further experiments as only lower amounts of siRNA were applied so far which showed up to be inefficient.

Therefore, for full comparison of the three types of PEI, experiments at the efficient final amount of siRNA (36 pmol) with polyplexes composed of either BPEI, c-LPEI and LPEI all in range of N/P ratios could be carried out in future. Also, a range of siRNA amounts would be worth to examine as in published studies also 25 pmol siRNA per well was proved as efficient (Grayson et al. 2006) and already 38 pmol led to PEI toxicity (Oskuee et al. 20).

Regarding the parameters of polyplex formation, namely different buffer (HBS or HBG) and incubation time after polyplexing, they are both related to polyplex size, colloidal stability and aggregation. Wightman et al. (2001) compared BPEI (25 kDa)/pDNA and LPEI (22 kDa)/pDNA polyplex formation in salt conditions (HBS and 0,5 x HBS) and in 5 % glucose which is similar to conditions used in this thesis: 20 mM HEPES/150 mM NaCl (HBS) and 20 mM HEPES/5 % (w/V) glucose (HBG). While in 0,5 x HBS buffer both types of complexes tend to grow with time, especially LPEI (22 kDa)/pDNA formed large aggregates, in 5 % glucose both complexes remained small. In that study, *in vitro* transfection efficiency was highest for the largest complexes and low for the complexes created in 5 % glucose. Taschauer et al. (2016) explain that a stabilizing effect on physiochemical properties of polyplexes showed by HBG can, on the other hand, lead to reproducible quality of formed polyplexes. Results of this thesis did not show any apparent difference in efficiency of polyplexes created in either HBS or HBG as well as with or without applying incubation time after polyplexing. The experiments with efficient knockdown providing also comparison of these parameters (Figure 24) were repeated twice, however, as the results for c-LPEI-based polyplexes at 36 pmol siRNA per well indicated slight difference for those parameters (Figure 26), to make a conclusion it would be optimal to repeat the experiments furthermore.

Further, Layer-by-Layer assembled gold nanoparticles were tested for siRNA delivery with aim to perform pilot *in vitro* experiments with the nanoparticles. The nanoparticles compose of different layers, briefly, gold nanoparticles represent a core coated with one layer of PEI, layer of siRNA follows and the terminal layer is again PEI. Their potential in siRNA delivery is explained by Elbakry et al. (2009). Compared to intensively investigated self-assembled aggregates of polymer and nucleic acid, e.g. polyplexes, of which formation leads generally to heterogenous systems, Layer-by-Layer strategy of coating monodisperse gold nanoparticles could result in formation of carriers that remains

monodisperse. Also, AuNPs were already used to deliver siRNA as presented e.g. by Giljohann et al. (2009). Although they achieved gene silencing, it manifested only after 4 days. Since polyethylenimine has some great properties for nucleic acid delivery, as described in section 1.2 - 1.4., Layer-by-Layer strategy of coating AuNPs with polyethylenimine and siRNA could result in efficient carrier.

Elbakry et al. (2009) tested these nanoparticles containing BPEI (25kDa) with following layers: AuNP-BPEI-siRNA-BPEI. Interestingly, they achieved gene silencing efficiency without apparent toxicity. Compared to this, the same formulation in our experiments was not capable of gene silencing without toxic effects on cells. However, only pilot experiments were carried out resulting in specifying of range of concentrations ($1x10^8 – 2{,}5x10^8$ particles/well) in which the toxicity showed to be lower than in the highest concentrations and gene silencing ability was indicated. Therefore, future experiments can build up on these findings.

As Elbakry et al. (2009) suggested, fabrication of the nanoparticles with biodegradable polymer could result in improved intracellular release of the cargo. We examined AuNP-BPEI-siRNA-c-LPEI and AuNP-c-LPEI-siRNA-c-LPEI nanoparticles, however the first showed similar results as AuNP-BPEI-siRNA-BPEI and not achieved knockdown without apparent toxicity. Interestingly, the AuNP-c-LPEI-siRNA-c-LPEI nanoparticles showed in the concentration in range of 10^8 particles/well no apparent toxicity, compared to the two other types of particles already toxic in such concentrations, which can correlate with lower toxicity of c-LPEI compared to BPEI. However, also no gene silencing was achieved at this concentration. Similarly as aforementioned, future experiments built up on established findings can be performed.

Several knockdown studies are frequently presented in current literature, however with different methods of data presentation. Three relatively often used methods, described in section 3.8.1, are compared here with highlighting their advantages and disadvantages.

If using Method 1, luciferase activity (in RLU) of samples (treated with nanoparticles carrying LucsiRNA or NCsiRNA) is calculated relative to untreated cells. Total protein content of samples determined by BCA assay is not taken into calculation. Although this method of presenting the luciferase gene knockdown is often used in literature (Zintchenko et al. 2008, Oskuee et al. 2010, Liu et al. 2011) it is necessary to carefully interpret a luciferase expression decrease of samples which can be caused not only

by gene knockdown but also by carrier toxicity. Thus, knockdown is only indicated if the decrease is for LucsiRNA but not for NCsiRNA or if the decrease for LucsiRNA is markedly greater, then the knockdown is represented by the difference.

In Method 2, luciferase activities (in RLU) of samples (treated with nanoparticles carrying LucsiRNA or NCsiRNA) as well as of untreated cells are normalized by their total protein content determined by BCA assay resulting in RLU/µg. These values are then presented as percentage of values of untreated cells. This method of presenting the luciferase knockdown, used for example by Hattori et. al. (2014), advantageously decouple a potential toxicity of a carrier from the knockdown thanks to the normalization. However, as a potential disadvantage of combination the data from two assays, based on the experience when processing the data of this thesis, results presented by this method often give large error bars.

For Method 3, which is presented e.g. by Schäfer et al. (2010), it is assumed that negative control formulation (carrying NCsiRNA) has the same possible toxicity or any other influence on cell growth and viability as the formulation tested (carrying LucsiRNA). Anyway, in this thesis, the judgement on same toxicity was done also based on total protein content determined by BCA assay. If problems with protein content determination occurred or if the protein content of sample treated with nanoparticle carrying LucsiRNA was not comparable to sample with negative control formulation, the Method 3 was considered as inappropriate as the decrease in the luciferase activity of the formulation tested could be easily misinterpreted because of uncertainty the knockdown is the cause of the decrease.

6. CONCLUSION

In this study, the aim was *in vitro* evaluation of the ability of different types of polyethylenimine-based nanoparticles to deliver pDNA or siRNA.

For pDNA delivery, polyplexes based on linear polyethylenimine (LPEI) labelled with gadoteric acid (LPEI-DOTA-Gd) were examined to evaluate an effect of the labelling on the LPEI transfection ability. The transfection efficiency was determined in comparison with polyplexes based on unlabeled LPEI, with the result showing the transfection ability of LPEI-DOTA-Gd is comparable to that of LPEI. Thereby indicating the incorporation of DOTA-Gd into LPEI did not affect its DNA transfection ability.

For siRNA delivery, polyplexes and Layer-by-Layer assembled gold nanoparticles were tested for their gene silencing efficiency.

Polyplexes mediated siRNA delivery studies focused on polyplexes based on three types of polyethylenimine: linear (LPEI), branched (BPEI) and disulfide crosslinked linear (c-LPEI), and on evaluating their gene silencing efficiency under different polyplex parameters and at different concentrations. The efficient gene silencing was achieved with BPEI-based polyplexes of N/P ratio 9 at final siRNA amount 36 pmol/well. Those polyplexes were efficient when created both in HBG or HBS as well as with or without applying incubation time after polyplexing. At lower siRNA amount (1 pmol, 5 pmol and 10 pmol siRNA/well) BPEI-based polyplexes showed inefficient knockdown. All three types of PEI were tested for polyplexes mediated siRNA delivery at 5 pmol siRNA/well where, however, all showed no gene silencing efficiency. The aim was also to find optimal incubation conditions for the delivery. The following were the optimized conditions for knockdown: MDA MB-231-PGK-eGPF-Luc cells incubated for 48 h with the polyplexes when starting the incubation in basal media and after 4 h by adding the complete media (+ FBS, + L-Glu).

Three types of Layer-by-Layer assembled gold nanoparticles containing in the layers BPEI or c LPEI were tested for siRNA delivery. AuNP-BPEI-siRNA-BPEI nanoparticles were tested at range of concentrations 10^5-10^9 particles/well. While in the lower concentrations (10^5-10^7 particles/well) they showed inefficient gene silencing, in the higher concentrations (10^8-10^9 particles/well) they caused toxic effects on cells. However, in concentration range $1x10^8$ – $2{,}5x10^8$ particles/well the toxicity was lower than

in the highest concentrations and gene silencing ability was indicated, which can be used as a starting point for future studies. AuNP-BPEI-siRNA-c-LPEI nanoparticles were tested at concentrations 10^5-10^8 particles/well with similar results. AuNP-c-LPEI-siRNA-c-LPEI nanoparticles were tested only at concentration in range of 10^8 particles/well and the results showed no gene silencing efficiency as well as no apparent toxicity.

8. ABBREVIATIONS

AAV	adeno-associated viruses
AGO2-RISC	complex of Argonaut 2 and RNA-induced silencing complex
AIDS	acquired immune deficiency syndrome
AMD	age-related macular degeneration
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCA	bicinchoninic acid
bp	base pairs
BPEI	branched polyethylenimine
BSA	bovine serum albumin
CCLR	Cell Culture Lysis Reagent
CO_2	carbon dioxide
c-LPEI	crosslinked linear polyethylenimine
D-LH_2	D-luciferin
DME	diabetic macular edema
DNA	deoxyribonucleic acid
DOTA-Gd	gadoteric acid
DPBS	Dulbecco's phosphate buffered saline
ECACC	European Collection of Authenticated Cell Cultures
FBS	fetal bovine serum
GSH/GSSG	glutathione (reduced/oxidized form)
HBG	HEPES buffered glucose
HBS	HEPES buffered saline
kDa	kilodalton
LB	Luciferin Buffer
LAB	Luciferase Assay Buffer
LF	Lipofectamine® RNAiMAX Reagent (or its lipoplexes)
L-Glu	L-Glutamine
LMW	low molecular weight
LPEI	linear polyethylenimine
LPEI-DOTA-Gd	LPEI labeled with gadoteric acid
LPL	lipoprotein lipase

LucsiRNA	small interfering RNA targeted against Firefly luciferase gene
miRNA	microRNA
MMCT	Laboratory of MacroMolecular Cancer Therapeutics
MQ-water	MiliQ water (prepared fresh in the Millipore device by reverse osmosis)
MRI	magnetic resonance imaging
MW	molecular weight
NADPH/NADP+	nicotinamide adenine dinucleotide phosphate (reduced/oxidized form)
negCTRL	negative control
NCsiRNA	non-targeted negative control small interfering RNA
N/P ratio	molar ratio of nitrogens from the polyethylenimine and phospates from nucleic acid
ODNs	antisense oligodeoxynucleotides
PAMAM	polyamidoamine
pDNA	plasmid DNA
PEI	polyethylenimine
PEG	polyethylene glycol
pre-mRNA	pre-messenger RNA
P/S	penicillin/streptomycin
PPi	pyrophosphate
PPI	polypropylenimine
RLU(s)	relative light unit(s)
RNAi	RNA interference
siRNA	small interfering RNA
SNALP	stable nucleic acid lipid particle
TRXred/TRXox	thioredoxin (reduced/oxidized form)
UT	untreated cells

9. LIST OF TABLES

10. LIST OF FIGURES

11. REFERENCES

Badr C. E. and Tannous B. A. Bioluminescence imaging: progress and applications. *Trends in Biotechnology,* 2011; 29(12): 624-33.

Breunig M., Lungtwitz U., Liebl R. and Goepferich A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. *Proceedings of the National Academy of Sciences of the United States of America,* 2007; 104(36): 14454-59.

Breunig M., Hozsa C., Lungwitz U., Watanabe K., Umeda I., Kato H. and Goepferich A. Mechanistic investigation of poly(ethylene imine)-based siRNA delivery: Disulfide bonds boost intracellular release of the cargo. *Journal of Controlled Release,* 2008; 130: 57-63.

Cai Y., Yu X., Hu S. and Yu J. A brief review on the mechanisms of miRNA regulation. *Genomics Proteomic Bioinformatics.* 2009; 7(4): 147-54.

Dai F. and Liu W. Enhanced gene transfection and serum stability of polyplexes by PDMAEMA-polysulfobetaine diblock copolymers. *Biomaterials,* 2011; 32: 628-38.

Conti E., Franks N. P. and Brick P. Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes. *Structure,* 1996; 4(3): 287-98.

Dash P. *University of Birmingham. Standard Protocols: Luciferase Assay* [online]. Unknown date. [cited. 2017-03-19]. Available from: http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=2507).

Elbakry A., Zaky A., Liebl R., Rachel R., Goepferich A. and Breunig M. Layer-by-Layer assembled gold nanoparticles for siRNA delivery. *Nano letters,* 2009, 9(5): 2059-64.

Fan F. and Wood K. V. Bioluminescent assays for high-throughput screening. *ASSAY and Drug Development Technologies*. 2007; 5(1): 127-36.

Giljohann D. A., Seferos D. S, Prigodich A. E., Patel P. C and Mirkin C. A. Gene Regulation with Polyvalent siRNA-Nanoparticle Conjugates. *Journal of the American Chemical Society,* 2009; 131(6): 2072-3.

Grayson A. C. R., Doody A. M. and Putnam D. Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery *in vitro*. *Pharmaceutical Research*, 2006; 23(8): 1867-76.

Greer L. F. and Szalay A. A. Imaging of light emission from the expression of luciferases in living cells and organisms: a review. *Luminescence,* 2002; 17: 43-74.

Hattori Y., Nakamura A., Arai S., Nishigaki M, Ohkura H., Kawano K., Maitani Y. and Yonemochi E. *In vivo* siRNA delivery system for targeting to the liver by poly-L-glutamic acid-coated lipoplex. *Results in Pharma Sciences,* 2014; 4: 1–7.

Ibraheem D., Elaissari A. and Fessi H. Gene therapy and DNA delivery systems. *International Journal of Pharmaceutics,* 2014; 459: 70-83.

Jacobsen L. B., Calvin S. A., Colvin K. E. and Wright M. FuGENE 6 Transfection Reagent: the gentle power. *Methods,* 2004; (33): 104-12.

Jin L., Zeng X., Liu M., Deng Y. and He N. Current progress in gene delivery technology based on chemical methods and nano-carriers, *Theranostics,* 2014; 4(3): 240-55.

Kaskova Z. M., Tsarkova A. S. and Yampolsky I. V. 1001 lights: luciferins, luciferases, their mechanisms of action and applications in chemical analysis, biology and medicine. *Chemical Society Reviews,* 2016; 45: 6048-77.

Kesharwani P., Gajbhiye V. and Jain N. K. A review of nanocarriers for the delivery of small interfering RNA. *Biomaterials,* 2012; 33: 7138-50.

Kichler A., Leborgne C., Coytaux E. and Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. *The Journal of Gene Medicine,* 2001; 3: 135-44.

Koide H., Okamoto A., Tsuchida H., Ando H., Ariizumi S., Kiyokawa C., Hashimoto M., Asai T., Dewa T. and Oku N. One-step encapsulation of siRNA between lipid-layers of multi-layer polycation liposomes by lipoplex freeze-thawing. *Journal of Controlled Release,* 2016; 228: 1–8.

Kwok A. and Hart S. L. Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery. *Nanomedicine: Nanotechnology, Biology and Medicine,* 2011; 7: 210-19.

Lächelt U and Wagner E. Nucleic acid therapeutics using polyplexes: A journey of 50 years (and beyond). *Chemical Reviews,* 2015; 115: 11043-78.

Liu Y., Samsonova O., Sproat B., Merkel O. and Kissel T. Biophysical characterization of hyper-branched polyethylenimine-graftpolycaprolactone-block-mono-methoxyl-poly(ethylene glycol) copolymers (hy-PEI-PCL-mPEG) for siRNA delivery. *Journal of Controlled Release*, 2011; 153: 262–68.

Lorenzer C., Dirin M., Winkler A., Baumann V. and Winkler J. Going beyond the liver: Progress and challenges of targeted delivery of siRNA therapeutics. *Journal of Controlled Release,* 2015; 203: 1-15.

Magnusson T, Haase R, Schleef M, Wagner E and Ogris M. Sustained, high transgene expression in liver with plasmid vectors using optimized promoter-enhancer combinations. *Journal of Gene Medicine*, 2011; 13: 382-91.

Mattes J., Yang M. and Foster P. S. Regulation of microRNA by antagomirs: a new class of pharmacological antagonists for the specific regulation of gene function? *American journal of respiratory cell and molecular biology*, 2007; 36(1), 8-12.

Marques S. M. and Esteves da Silva J. C. G. Firefly bioluminescence: A mechanistic approach of luciferase catalyzed reactions. *Life*, 2009; 61(1): 6-17.

Meyer M., Philipp A., Oskuee R., Schmidt C. and Wagner E. Breathing life into polycations: Functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery. *Journal of American Chemical Society,* 2008; 130: 3272-3.

Müller Katharina: *Luciferase based reporter gene assays for investigating nucleic acid delivery in vitro,* 2017. Diploma thesis. University of Vienna, Vienna,140 pages.

Nguyen J. and Szoka F. C. Nucleic acid delivery: The missing pieces of the puzzle? *Accounts of chemical research,* 2012; 45(7): 1153-62.

Oskuee R. K., Philipp A., Dehshahri A., Wagner E and Ramezani M. The impact of carboxyalkylation of branched polyethylenimine on effectiveness in small interfering RNA delivery. *Journal of Gene Medicine,* 2010; 12: 729–738.

Ozcan G., Ozpolat B., Coleman R. L., Sood A. K. and Lopez-Berestein G. Preclinical and clinical development of siRNA-based therapeutics. *Advanced Drug Delivery Reviews,* 2015; 87: 108-19.

Patrick M. *Market Realist. Alnylam Pharmaceuticals Advances Its Patisiran Research Program*. [online]. 02.2017 [cited 2017-04-18]. Available from: http://marketrealist.com/2017/02/alnylam-pharmaceuticals-continues-advance-patisiran-research-program-2017/.

Peng Q., Zhong Z. and Zhuo R. Disulfide cross-linked polyethylenimines (PEI) prepared via thiolation of low molecular weight PEI as highly efficient gene vectors. *Bioconjugate Chemistry,* 2008; 19: 499-506.

Pinel S., Aman E., Erblang F., Dietrich J., Frisch B., Sirman J., Kichler A., Sibler A., Dontenwill M., Schaffner F. and Zuber G. Quantitative measurement of delivery and gene silencing activities of siRNA polyplexes containing pyridylthiourea-grafted polyethylenimines. *Journal of Controlled Release,* 2014; 182: 1-12.

Promega. Dual-Luciferase® Reporter Assay System [online]. Revised at 09.2016 [cited 2017-03-19]. Available from: https://at.promega.com/-/media/files/resources/protocols/technical-manuals/0/dual-luciferase-reporter-assay-system-protocol.pdf.

Roche Molecular Biochemicals. FuGENE 6 Transfection Reagent Instruction Manual, Version 5 [online]. 08.2001 [Cited at 2017-04-25]. Available from: http://www.genesil.com/docs/FuGENE%206%20(Roche)%20transfection%20reagent%20instruction.pdf.

Schäfer J., Höbel S., Bakowsky U. and Aigner A. Liposome-polyethylenimine complexes for enhanced DNA and siRNA delivery. *Biomaterials,* 2010; 31: 6892-900.

Scholz C. and Wagner E. Therapeutic plasmid DNA versus siRNA delivery: Common and different tasks for synthetic carriers. *Journal of Controlled Release,* 2012; 161: 554–65.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J. and Klenk D. C. Measurement of protein using bicinchoninic acid. *Analytical Chemistry,* 1985; 150: 76-85.

Son S., Namgung R., Kim J., Singha K. and Kim W. J. Bioreducible polymers for gene silencing and delivery. *Accounts of chemical research,* 2011; 45(7): 1100-112.

Taranejoo S., Liu J., Verma P. and Hourigan K. A review of the developments of characteristics of PEI derivatives for gene delivery applications. *Journal of Applied Polymer Science,* 2015; 132(25): 1-8.

Tarcha P. J., Pelisek J., Merdan T., Waters J., Cheung K., von Gersdorff K., Culmsee C. and Wagner E. Synthesis and characterization of chemically condensed oligoethylenimine containing beta-aminopropionamide linkages for siRNA delivery. *Biomaterials,* 2007; 28: 3731-40.

Thermo Fisher Scientific. Luciferase Reporters [online]. Revised at 04.2017 (2017a) [cited 2017-04-17] Available from: https://www.thermofisher.com/cz/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/luciferase-reporters.html.

Thermo Fisher Scientific. Reporter Gene Assays [online]. Revised at 04.2017 (2017b) [cited 2017-04-17]. Available from: https://www.thermofisher.com/cz/en/home/references/gibco-cell-culture-basics/transfection-basics/reporter-gene-assays.html.

Thermo Fisher Scientific. Factors Influencing Transfection Efficiency [online]. Revised at 04.2017 (2017c) [cited at 2017-04-25]. Available from: https://www.thermofisher.com/cz/en/home/references/gibco-cell-culture-basics/transfection-basics/factors-influencing-transfection-efficiency.html.

Taschauer A., Geyer A., Gehrig S., Maier J., Sami H. and Ogris M. Up-scaled synthesis and characterization of nonviral gene delivery particles for transient *in vitro* and *in vivo* transgene expression. *Human Gene Therapy Methods,* 2016, 27(3):87-97.

Tros de Ilarduya C. and Düzgüneş N. Efficient gene transfer by transferrin lipoplexes in the presence of serum. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 2000; 1463(2): 333-42.

Wightman L., Kircheis R., Rössler V., Carrota S., Ruzicka R., Kursa M. and Wagner E. Different behaviour of branched and linear polyethylenimine for gene delivery *in vitro* and *in vivo*. *The Journal of Gene Medicine,* 2001; 3: 362-72.

Yin H., Kanasty R. L., Eltoukhy A. A., Vegas A. J., Dorkin J. R and Anderson D. G. Non-viral vectors for gene-based therapy. *Nature Review Genetics,* 2014; 15: 541-55.

Zintchenko A., Philipp A., Dehshahri A. and Wagner E. Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. *Bioconjugate Chemistry,* 2008; 19: 1448–55.