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„Optimization of multiplexed assays for in vitro splice correction and cytotoxicity“

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Stefanie Kaufmann, BSc

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Table of contents

Declaration

I hereby certify that I am the sole author of this thesis and have not used any aids other than those specified. The use of other sources is clearly acknowledged and indicated. Neither ChatGPT nor other artificial intelligence was used for research in this thesis.

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I. Abstract

Splice switching oligonucleotides (SSO) can alter pre-mRNA transcripts by interacting with the splicing machinery to restore defective or absent proteins, which would otherwise cause diseases. Cell based *in vitro* assays are essential tools for the development of these SSO-based RNA targeted therapeutics for studying their transfection efficiency and cell viability profile. Polyethylenimine (PEI) based polymers work efficiently as transfection agents by complexing nucleic acids, like SSOs, and offer diversity in structure and available functionalities. Towards this, the present thesis includes cell-based assays for studying different versions of PEI based SSO polyplexes for transfection efficiency and cell viability profile on human cervical cancer cell line HeLa pLuc705 and primary endothelial cells of porcine origin (PEC). It was important to first optimize resazurin assay for both cell types before examining cytotoxicity of polyplexes. HeLa pLuc705 and PEC were seeded at different cell numbers and resazurin assay was carried out by fluorometric readout of the supernatant. Freshly prepared housemade resazurin solution and commercially available Alamar Blue HS reagent was compared regarding their sensitivity, reproducibility and different modes of reading.

SSO-based polyplexes were prepared from different PEI polymers with molecular weights ranging from 1 to 3 kDa and different degrees of disulfide crosslinking. As positive non-crosslinked controls branched PEI 25 kDa (BPEI) and lipid-based commercially available Lipofectamine 3000 were used. The cationic polymers were used to complex luciferase SSO (LucSSO) or negative SSO (NegSSO), which were then used in two concentrations, 160 pmol and 80 pmol for the treatment of PECs (for studying cell viability via resazurin assay) and HeLa pLuc705 cells (for studying splice correction by firefly luciferase assay). In the present study, it could be shown that using the supernatant of polyplex treated cells, cell viability can be simultaneously assayed along with luciferase activity readout from the cells, providing a time-effective and sensitive multiplexed method for combining cell viability with transfection studies.

II. Zusammenfassung

„Splice switching“-Oligonukleotide (SSO) können prä-mRNA-Transkripte durch Wechselwirkungen mit der Spleißmaschinerie verändern, um defekte oder fehlende Proteine wiederherzustellen, die andernfalls Krankheiten verursachen würden. Zellbasierte In-vitro-Assays sind wesentliche Instrumente für die Entwicklung dieser SSO-basierten RNA-gerichteten Therapeutika zur Untersuchung ihrer Transfektionseffizienz und ihres Zellviabilitätsprofils. Polymere auf der Basis von Polyethylenimin (PEI) mit unterschiedlichen Strukturen und Funktionalitäten wirken als effiziente Transfektionsmittel, indem sie Nukleinsäuren wie SSOs komplexieren und in die Zelle transferieren. In der vorliegenden Arbeit wurden zellbasierte Assays zur Untersuchung verschiedener Versionen von PEI-basierten SSO-Polyplexen auf ihre Transfektionseffizienz und ihr Effekt auf die Zellviabilität an der menschlichen Gebärmutterhalskrebs-Zelllinie HeLa pLuc705 und an primären Endothelzellen porcinen Ursprungs (PEC) durchgeführt. Es war wichtig, zunächst den Resazurin-Assay für beide Zelltypen zu optimieren, bevor die Zytotoxizität der Polyplexe untersucht wurde. HeLa pLuc705 und PEC wurden in unterschiedlichen Zellzahlen ausgesät und der Resazurin-Assay wurde durch fluorometrische Auslesung des Überstands und anschließende Ermittlung einer Kalibrationsgerade durchgeführt. Frisch hergestellte Resazurinlösung und handelsübliches Alamar Blue HS-Reagenz wurden hinsichtlich ihrer Empfindlichkeit, Reproduzierbarkeit und unterschiedlichen Ablesemodi verglichen.

SSO-basierte Polyplexe wurden aus PEI-Polymeren hergestellt, deren Molekulargewicht zwischen 1 bis 3 kDa liegt und die einen unterschiedlichen Grad an Disulfidbrücken aufweisen. Als positive, nicht quervernetzte Kontrollen wurden verzweigtes PEI (BPEI) und Lipid-basiertes handelsübliches Lipofectamine 3000 verwendet. Die kationischen Polymere wurden verwendet, um Luciferase-SSO (LucSSO) oder negative SSO (NegSSO) zu komplexieren, die dann in zwei Konzentrationen, 160 pmol und 80 pmol, für die Behandlung von PECs (zur Untersuchung der Zellviabilität mittels Resazurin-Assay) und HeLa pLuc705-Zellen (zur Untersuchung der Spleißkorrektur mittels Firefly Luciferase Assay) verwendet wurden. In der vorliegenden Studie konnte gezeigt werden, dass mit dem Überstand der polyplexbehandelten Zellen die Zellviabilität gleichzeitig mit der Luciferase Expression der Zellen untersucht werden kann, was eine zeitsparende und empfindliche Multiplex-Methode zur Kombination von Zelllebensfähigkeit und Transfektionsstudien darstellt.

1. Aim of the thesis

The thesis focused on the overall goal of simultaneously detecting *in vitro* splice-correction and cytotoxicity from the same transfection study i.e. developing multiplexed protocol for combining transfection and cytotoxicity readout of different polyethylenimine (PEI) based SSO-polyplexes. Different PEI polymers included- linear PEI 3kDa (LPEI3kDa), crosslinked PEI 0.5 (C-LPEI0.5), crosslinked PEI 1.0 (C-LPEI1.0), crosslinked PEI HB (C-LPEI HB) and as positive non-crosslinked controls branched PEI (BPEI). Towards this, the aims were as follows:

a. Optimizing cell viability assays

The first aim was to study the stability of resazurin solution made from resazurin natrium salt and phosphate buffered saline. The second aim was to compare two different modes of reading, namely bottom and top reading using housemade fresh resazurin solution and two commercial products (VWR and Alamar Blue HS). The third aim was to compare the freshly prepared resazurin solution to commercially available Alamar Blue HS regarding their sensitivity and reproducibility and to find out the most effective and economical method.

b. Cytotoxicity profile of different PEI-SSO polyplexes in porcine endothelial cells

Different PEI-SSO polyplexes, namely, linear PEI 3kDa (LPEI3kDa), crosslinked PEI 0.5 (C-LPEI0.5), crosslinked PEI 1.0 (C-LPEI1.0), crosslinked PEI HB (C-LPEI HB) and as positive non-crosslinked controls branched PEI (BPEI), were prepared and the aim was to investigate their toxicity in porcine endothelial cells by using the optimized version of cell viability assay.

c. Multiplexing: combining splice correction readout of firefly luciferase assay with cell viability assay in HeLa pLuc705 cells

The first goal for investigating splice-correction in HeLa pLuc705 cell model was to determine the basal expression level of luciferase in HeLa wt and HeLa pLuc705 via firefly luciferase assay and conducting BCA assay-based protein estimation to obtain normalization of luciferase expression. The next aim was to investigate splice-correction by different PEI-SSO polyplexes consisting of luciferase splice-switching oligonucleotides complexed with cationic polymers, namely LPEI3kDa, C-LPEI 0.5, C-LPEI 1.0, C-LPEI HB, Lipofectamine 3000 and BPEI in two different concentrations (80 pmol and 160 pmol) compared to the control negative SSO, which contains a scrambled sequence of the luciferase gene. The final aim was to multiplex the optimized cell viability assay with firefly luciferase assay to detect rate of splice correction and cytotoxicity simultaneously.

2. Introduction

2.1. Nucleic acid therapy

In the last two decades Gene therapy became a promising way of treatment of hereditary diseases like Alzheimer's disease, β-thalassaemia and Duchenne muscular atrophy. The common goal in treatment was to modify genetic information with exogenous DNA or RNA, consequently exchanging defective genes with the corresponding and corrected counterpart or deleting mutated nucleotides which are the cause for inherited and often incurable diseases. This concept of therapy enables the usage of nucleic acids *in vivo,* to achieve better symptoms or even cure of the disorder in patients. Since mutations can cause multiple pathological conditions, nucleic acid therapeutics like DNA or RNA as well as small interfering RNA (siRNA), antisense oligonucleotides (ASO), small hairpin RNA (shRNA) and plasmid DNA (pDNA) are outstandingly appealing for the treatment of cancer and other hereditary illnesses (Chong et al. 2021).

One of the advantages of nucleic acid therapeutics is that they ensure a high specificity to their desired target, but a challenge when developing gene-based drugs is their formulation to achieve this effect. Naked nucleic acids cannot be applied to a tissue or organism effectively, because of their negative charge and high molecular weight. Additionally, nucleases in serum degrade DNA and RNA, resulting in a half-life of approximately ten minutes (Yang et al. 2014).

At present, two main ways of transfection exist, viral and non-viral transfections. Non-viral methods include physical/mechanical, as well as chemical transfection, like lipid-based or non-lipid-based particles. See Figure 1 for further information. While viral transport is effective in transfecting cells, it offers many disadvantages in terms of cytotoxicity and immunogenic and even tumorigenic reactions (Walther und Stein 2000). Additionally, the risk of insertional mutagenesis is a constant problem when using inserting vectors such as the adeno-associated virus (Sant'Anna und Araujo 2022). In contrast, non-viral methods are less immunogenic and toxic compared to viral vectors. The choice for an optimal transfection strategy depends on factors including the type cells and the form of nucleic acids to be transfected (Li et al. 2021; Chong et al. 2021).

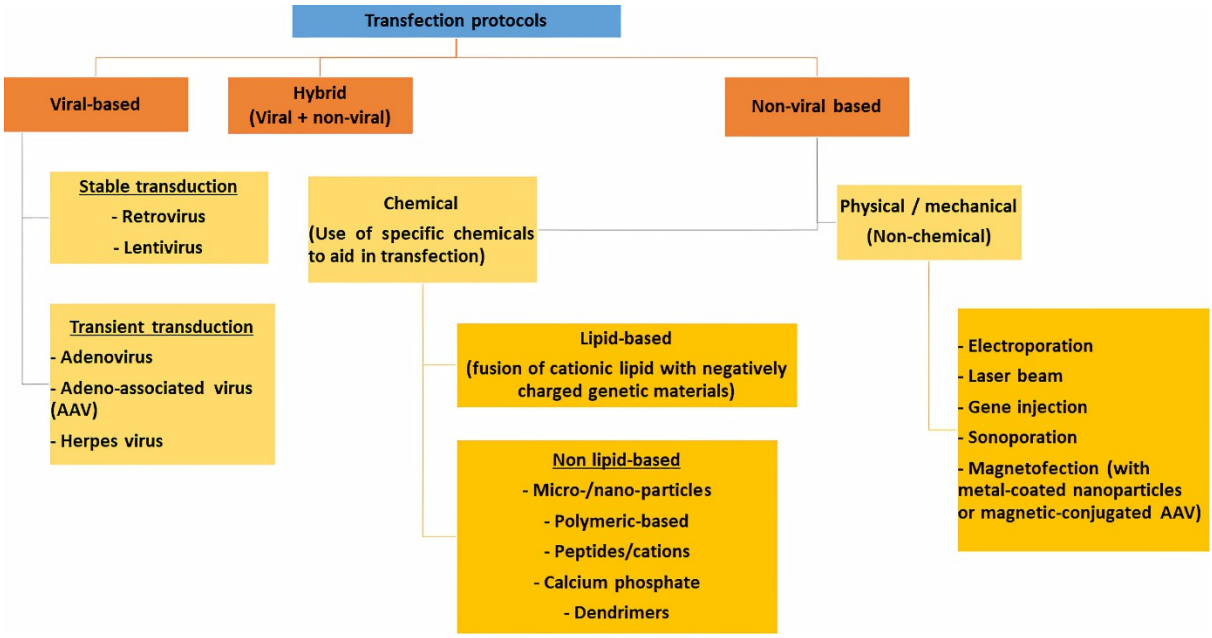

Figure 1. *Different methods of transfection*
Adapted from Chong et al.

Using ASOs in RNA therapy is a promising way in the treatment of above-mentioned neurological disorders. RNA therapeutics refer to the use of oligonucleotides to target various RNA for therapeutic efforts as well as in research to investigate the functions of specific genes.

Duchenne muscular atrophy, a muscle-wasting disease, is caused by nonsense or frameshift mutations of the DMD gene which expresses the functional protein Dystrophin (Okubo et al. 2020). Dystrophin is crucial to muscular function, because it preserves the integrity of muscle fibres and shields them from damage caused by contractions. The lack of dystrophin undermines the stability and function of muscle fibres, eventually resulting in muscle degeneration over time. (Elangkovan und Dickson 2021). In 2016, the first antisense oligonucleotide therapeutic for Duchenne muscular atrophy Eteplirsen got approved by the FDA, which aims to restore the defective reading frame and therefore lead to the expression of an abbreviated, but partly functional Dystrophin (Shimo et al. 2018). The sequence of Eteplirsen is complementary to exon51 of the dystrophin hnRNA. Thus, an RNA double strand is formed, which is not integrated into mature mRNA during splicing. By excluding the exon, the mutation-induced shift of the open reading frame is corrected again. This therapy enables to slow down the progression of the disease, but it is still not approved in EU by European Medicines Agency due to the low number of subjects and efficacy (Aartsma-Rus und Goemans 2019; CHMP 2018).

Another approved antisense drug is Nusinersen, which is used for mostly infant patients with spinal muscular atrophy (SMA). Spinal muscular atrophy is a rare autosomal recessive neuromuscular disorder that is caused by loss-of-function mutation of SMN1 gene and thus no expression of survival motor neuron (SMN) protein, resulting in progressive muscle waste. This ASO therapeutic modifies pre mRNA splicing of the SMN2 gene, a disease-modifying gene (in contrast to SMN1) and therefore induces an increase of SMN protein expressed. SMN2 gene is responsible for biosynthesis of low levels

of SMN protein, that cannot uphold the structure and function of motoneurons. The difference between SMN2 and SMN1 gene is the T>C substitution in exon 7, leading to increased skipping of it. Thus, Nusinersen prevents the exclusion of exon 7 causing the biosynthesis of functional and full-length SMN protein (Jablonka et al. 2022).

β-thalassaemia is a genetic blood disease which is characterized by a deficiency of β-globin chains, resulting in the decrease or absence of functional haemoglobin A expression. Several mutations in the introns 1 and 2 of β-globin-gene are known which cause this disorder. The mutation in intron 2, IVS2-705 which is characterized by a base change from T>G, leads to the creation of an aberrant 5' splicing site and activates a cryptic 3' site in intron 2 (Bauman et al. 2009). Based on this mutations a luciferase and EGFP reporter system could be established which is used in research for *in vitro* as well as *in vivo* studies (Guterstam et al. 2008; Sazani et al. 2002).

2.1.1. Antisense oligonucleotides

Antisense oligonucleotides are chemically synthesized oligonucleotides, usually 12-30 nucleotides in length that bind their target pre-mRNA by Watson-Crick base hybridization. Their high specificity is mainly due to the antisense nucleotide's length, allowing to target a specific RNA in cytoplasm as well as in the nucleus. Also the mechanisms of ASOs by which the target RNA is modified depends on their chemical properties, as well as the function of mRNA and its binding site for the ASO (Bennett 2019). Antisense oligonucleotides can be used either as single-stranded or double-stranded. The double-stranded ASOs use the RISC complex to degrade their target mRNA in the cytoplasm, whereas single-stranded ASOs have two main mechanisms of action, notably cleavage and degradation of RNA and occupancy-only mediated regulation, also known as steric blocking of translation (Khan et al. 2021), which will be discussed below. Some specific mechanisms are shown schematically in Figure 2.

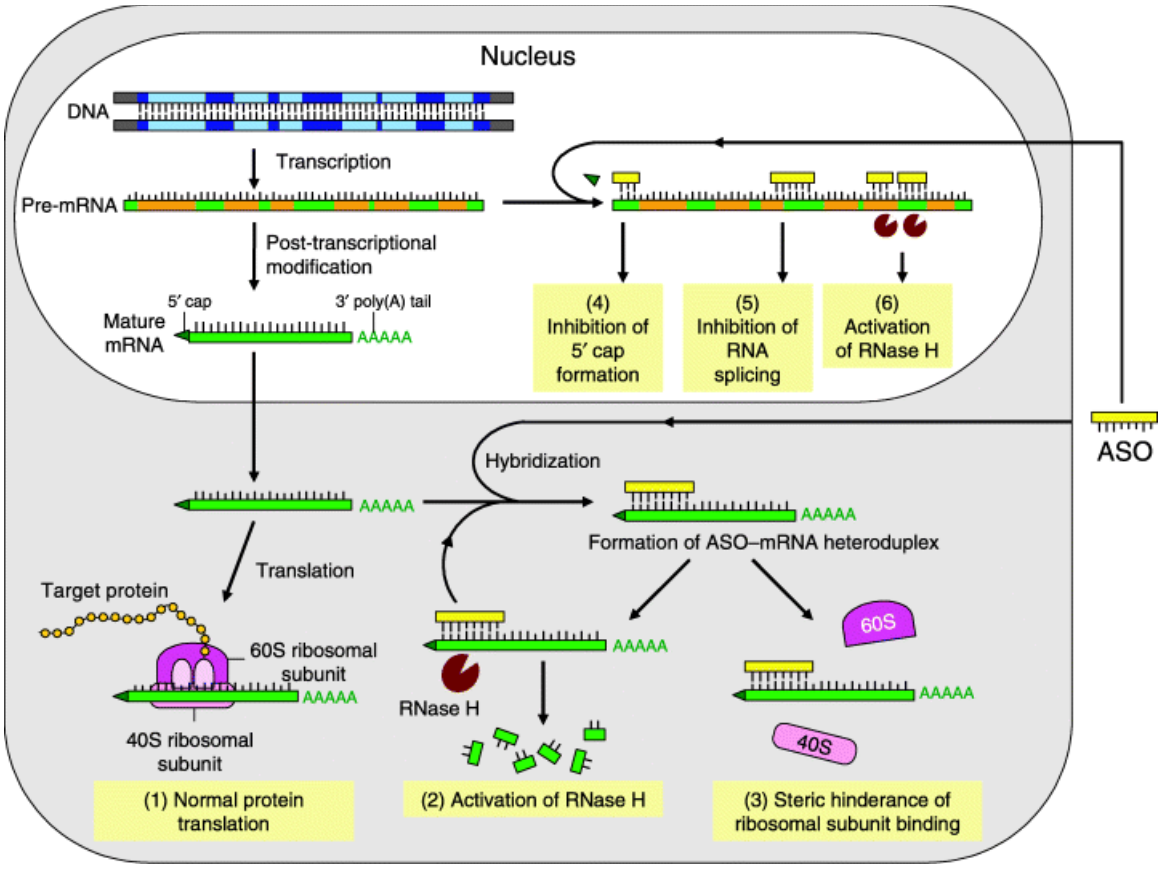

Figure 2. *Schematic overview of different mechanisms of action by antisense oligonucleotides.*
(1) Displays the translation of a functional protein in the absence of ASO. (2) and (6) RNase H degradation in cytoplasm and nucleus (3) steric hinderance of ribosomes (4) inhibition of 5'cap formation and (5) inhibition/alteration of RNA splicing. Retrieved from (Chan et al. 2006).

1. Cleavage and degradation of RNA

ASOs in the degradation pathway are characterized by cleavage either by RNase H1, siRNA-mediated AGO2 RISC complex or ribozymes-mediated cleavage.

RNase H is a ubiquitous nuclease in cytoplasm and nucleus in various mammalians, which is responsible for degrading RNA strand of an RNA-DNA-heteroduplex. There are two types of RNase H enzymes in humans, RNase H1 and RNase H2. ASOs bind their target mRNA first and then recruit RNase H1 to the duplex, subsequently leading to degradation of the RNA strand, leaving the ASO untouched and intact. For utilizing this mechanism of action, the oligonucleotides must consist of 5-7 DNA nucleotides (Crooke 1999; Bennett 2019)

2. Occupancy only mediated regulation

This mechanism occurs in cytoplasm as well as in the nucleus, enabling a diverse selection of possibilities to inhibit the expression of a specific protein on RNA level. "Steric blocking" for example inhibits the binding of ribosomal subunits on the target mRNA, thus making successful translation impossible. Steric hinderance can be accomplished in various other ways, like inhibition of polyadenylation and

blocking of microRNA to prevent it from binding their target RNA sequences, resulting in increased translation of the microRNA target. Another way of obtaining inhibition of RNA is to use splice-switching oligonucleotides that bind specific sequences on pre-mRNA and simultaneously acting as a hinderance for the spliceosome and its associated factors, which is the mechanism used in this thesis. After DNA transcription into pre-mRNA, a key step to generate mature mRNA is the splicing process. There, the exclusion of non-coding introns, as well as the in- and exclusion of specific exons is required to obtain a functioning protein. Small nuclear RNAs in the spliceosome are responsible for this mechanism (Crooke 1999). Alternative splicing enables a large diversity in proteins generated from one single mRNA, making it possible to achieve induction of isoform switching for expressing beneficial as well as inhibiting the expression of disease-associated proteins (Bauman et al. 2009).

a. Alternative splicing/splice switching

Splice-switching oligonucleotides offer a broad spectrum of applications e.g., correction of an aberrantly spliced transcript, production of a novel splice-variant that is not normally expressed or modify alternative splicing to change from one variant to another. Alternative splicing follows various patterns to achieve the desired isoform of a specific protein as demonstrated in Figure 3.

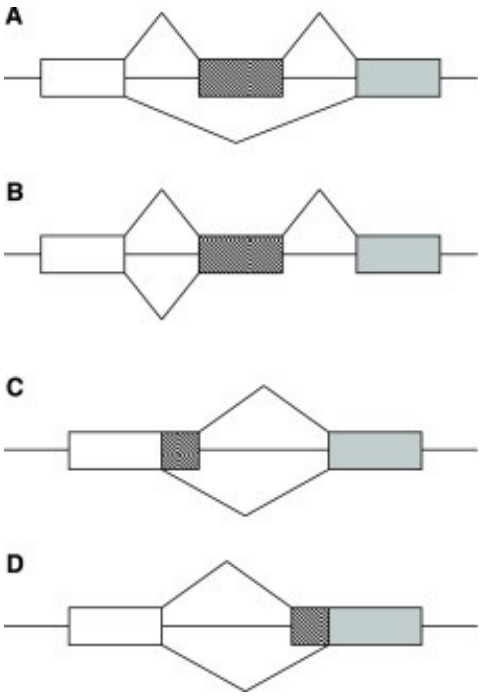

Figure 3. *Schematic overview of different patterns in alternative splicing.* (A) Exon skipping. (B) Intron retention. (C) Alternative 5' splice site. (D) Alternative 3' splice site. Adapted from (Bauman et al. 2009).

The big difference between splice-switching oligonucleotides and traditional antisense or RNAi is that the direction of splicing by SSOs requires that the target pre-mRNA bound to these compounds is not degraded by RNase H, like discussed above for common antisense mechanisms, which desire down-regulation of a specific gene (Bauman et al. 2009).

The underlying mechanism is based on downregulation of an unfavourable transcript while simultaneously upregulating expression of a preferred transcript Their activity is enhanced with increased target gene expression, because it enables increased creation of the desired splice variant. Luciferase expression only occurs when SSO successfully enters the cell, accesses pre-mRNA in the nucleus and binds the target sequence elements in the mutated intron, inducing correct splicing and exclusion of the defective intron (Bauman et al. 2009; Mercatante et al. 2002). Degradation of SSOs by nucleases can be prevented by modification of their backbone e.g., phopshorothioate (PS) or morpholino oligomers (PO).

The previously discussed antisense drug Eteplirsen for the treatment of Duchenne muscular atrophy restores the expression of a partly functioning Dystrophin protein via skipping the exon 51 in Dystrophin hnRNA. Nusinersen works similarly via the retention of inclusion of exon 7. The reporter system for splice-switching is based on a plasmid containing luciferase (or EGFP) gene with insertion of the mutated β-globin pre-mRNA. As mentioned above, intron 2 contains an aberrant splicing site on the 5'cap that activates a cryptic splicing site on 3' cap. Based on this, a mRNA transcript is generated that includes an intronic sequence coding for a stop codon, resulting in the expression of a truncated and defective β-globin protein (Bauman et al. 2009). Antisense oligonucleotides can be applied to exclude this intron, thus leading to correct splicing and expression of functional luciferase protein (Gusterstam et al. 2008).

2.1.2.Polyethylenimine based nucleic acid carriers

Common synthetic vector systems have been widely used for the delivery of nucleic acid, like as cationic polymers, cationic lipids and PAMAM dendrimers. The main advantages of using cationic polymers as a transfection agent are the relatively low toxicity compared to viral transfection, as well as their biodegradability and diversity in structures. Several studies have shown that transfection efficiency and cytotoxicity are clearly dependent on chemical properties, as well as the used cell line or organs. The ability to successfully deliver polyplexes is proved best by polyethylenimine (PEI), which has been thoroughly examined in the past was then considered as the highest standard for gene delivery (Breunig et al. 2007).

The positively charged amino groups of polyethylenimine interact electrostatically with the negatively charged phosphate groups of nucleic acids, thus enable condensation of both components into stable nanoparticles. This interaction is crucial to prevent the degradation of the nucleic acid during transport to the cell membrane where the uptake happens in several stages:

(1) Endocytosis via phagocytosis, macropinocytosis, micropinocytosis via clathrin- and calveolin-independent pathways or clathrin-mediated endocytosis takes place.

(2) Intracellular trafficking of endosomes regulated by various factors like Rab, SNARES and tethering proteins responsible for vesicular transport and membrane fusion.

(3) After this endosomal escape occurs during membrane fusion and is characterised by three effects, namely proton sponge-effect, umbrella effect and disruptive interaction with the endosomal membrane. The proton-sponge effect is based on the protonation of primary, secondary and tertiary titratable amino groups of cationic polymers. This causes an increase of the pH in the endosome/lysosome and results in enhanced proton production by the V-ATPase and an accompanied influx of chloride ions and water, ultimately causing swelling of the endosomal/lysosomal compartment. This swelling, also called umbrella effect leads to conformational changes and enlargement due to charge repulsion between positively charged amino groups. Subsequently, membrane disruption occurs supposedly due to interaction of unbound PEI with the endosomal phospholipid membrane. It has to be noted that only a small fraction of PEI polyplexes escapes the endosome before reaching the lysosome, as they are entrapped and undergo degradation.

(4) Lastly polyplexes entry the nucleus as their main target to enable genetic alteration, like previously described. The nuclear entry is actively regulated by nuclear pore mechanisms and passively via simple diffusion. The exact mechanism of nuclear import of PEI polyplexes is still unclear, whether the PEI polyplex gets disintegrated before the entry or if it is taken up as a whole (Benjaminsen et al. 2013; Juliano 2016; Casper et al. 2023; Hall et al. 2017).

Transfection efficiency of cationic polymers is dependent on intrinsic as well as environmental factors. Intrinsic influences are properties like polymer molecular weight, N/P ratio and structure of the polymer (linear, crosslinked or branched PEI), whereas environmental factors include ionic strength, competing polyanions and shear stress (Wang et al. 2019). Polyethylenimines have a high charge density and with increasing molecular weight the condensation with DNA, therefore stability of the complex is increased. A disadvantage of high molecular weight (HMW) PEI is that the intracellular release of the payload is decreased, resulting in less transfection efficiency, compared to low molecular weight (LMW) PEI. But in contrast, when the PEI's molecular weight is too low, polyplex stability decreases and the nucleic acid cannot be protected from degradation (Lü et al. 2016).

The most established PEIs as transfection agents (see Figure 4) are branched PEI (BPEI, 25 kDa) and linear PEI (LPEI, 22-25 kDa) having mid-range molecular weight. LPEI contains only secondary and limited primary amino groups whereas branched polyethylenimine may contain primary, secondary and tertiary amino groups, which enable electrostatic interactions with negatively charged molecules. Thus, BPEI has a stronger electrostatic interaction with DNA than LPEI, which means greater compaction, higher zeta potentials and smaller nanoparticle sizes at equivalent DNA concentrations (Lü et al. 2016). To improve transfection efficiency and simultaneously lower toxicity, cross-linking promised to be a reliable method to achieve these goals. These linkages e.g., disulfide-crosslinkers avoid disassembly during circulation and in extracellular spaces but can be cleaved easily to release the payload. Using disulfide bonds for crosslinking also enable the controlled release of nucleic acid in the cell (Wang et al. 2019). They lead to intracellular degradation, hydrolysis at low endosomal pH, enzymatic degradation, and cytosol-specific reduction by glutathione. Crosslinked polyethylenimines displayed high transfection efficiency and low cytotoxicity as a result of their rapid *in situ* degradation of

the polymer into small molecular weight and hydrophilic components, which are processed easily and removed from the cell (Das et al. 2015).

The cationic polymers discussed above are known for their cytotoxicity and potential side effects *in vivo* which is discussed in section 2.3.

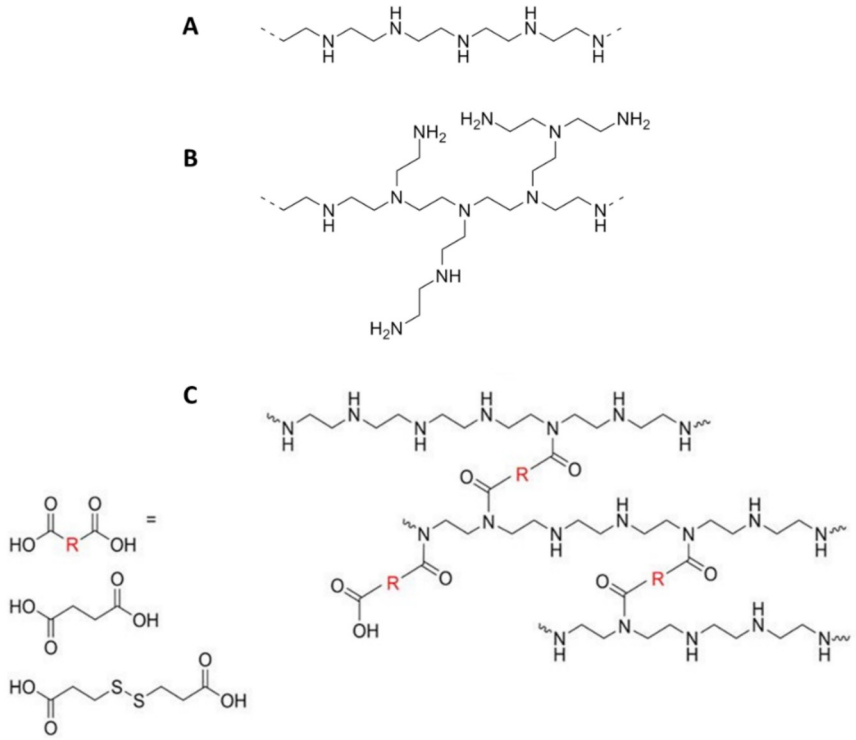

Figure 4. *Overview of different structures within the group of polyethylenimines*
(A) linear PEI (B) branched PEI (C) crosslinked PEI. Picture adapted from Bonner et al. and Wikipedia.org

The N/P ratio is defined as the ratio between the moles of the cationic polymer's amine groups (N) over the moles of the negatively charged phosphorus (P) of the nucleic acid. In general, the right N/P ratio is crucial for transfection, for example, a high N/P ratio indicates higher moles of nitrogen atoms, i.e. cationic polymer compared to the moles of phosphorus from the nucleic acid. Bonner et al. proved in their study, that a certain amount of free polymer leads to higher transfection efficiency. This indicates that the unbound fraction of PEI could be a key factor in the polyplexes' desirable escape from the endosome/lysosome system. To obtain fully complexed DNA a N/P ratio of 3 is needed minimum, but this low ratio is associated with low transfection ability (Wang et al. 2019).

2.2. Bioluminescence based reporters

Bioluminescence describes a special type of chemiluminescence and is found in a wide range of different species, from marine vertebrates and invertebrates, as well as in some fungi, microorganisms including some bioluminescent bacteria, and terrestrial arthropods such as fireflies. It conduces many natural purposes, as defence, camouflage, feeding or mating, especially for marine animals. Bioluminescence is characterized by an enzyme-catalysed and exothermic biochemical reaction that results in the emission of cold light (Lee 2008; Fan und Wood 2007). In this thesis, mainly firefly luciferase was used, a monomeric enzyme that requires no posttranslational modifications for its activity. There are various other luciferase enzymes like Renilla or Gaussia luciferase that are widely used in biomedical research. The structure of firefly luciferase was first determined in 1987 by J.R. de Wet in the north American beetle *Photinus pyralis* (England et al. 2016).

To achieve bioluminescence the enzyme luciferase reacts with the substrate D-luciferin in the presence of molecular oxygen, adenosine triphosphate (ATP) and magnesium to produce light, as shown schematically in Figure 5. In detail, it acts by combining luciferin with ATP, to form luciferyl-AMP as an enzyme-bound intermediate, which then reacts with O_2 to create the intermediate oxyluciferin in a high energy state. Subsequently, the energy transition to the ground state generates yellow-green light (550-570 nm) with a peak at 562 nm (Fan und Wood 2007).

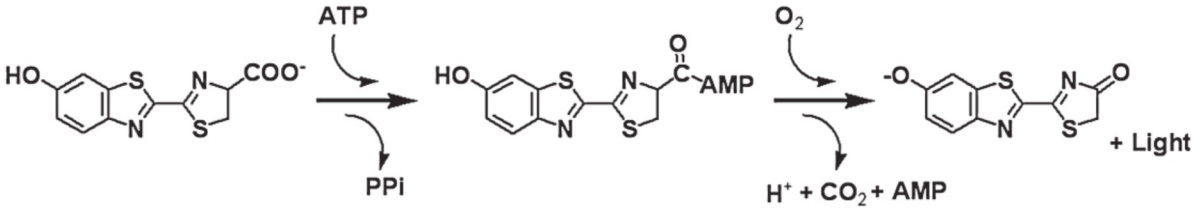

Figure 5. *Firefly luciferase catalyses the ATP-dependent oxidation of D-Luciferin* via an intermediate product to Oxyluciferin with light emission. Picture adapted from Fan und Wood.

Bioluminescent reporter gene assays have an advantage over fluorescent assays in that they are more sensitive when applied to complex biological samples, because the way of initial excitement of molecular orbitals into the excited state is not achieved via photons but chemical reactions. Due to the absence of photons, there is no inherent background signal like in fluorescent read-outs. This enables a precise measurement of small changes in light emission across a wide linear range (Fan und Wood 2007). A limitation of this reporter system may be that oxyluciferin is an intracellular protein remaining in the cytoplasm. Consequently, cell lysis is necessary for the measurement. Firefly luciferase reporter was used in this thesis to determine splice-correction of LucSSO treated HeLa pLuc705 which carry an aberrant luciferase gene which will be discussed in the following section.

2.2.1 Split luciferase model for splice correction

The reporter system used is a plasmid carrying a luciferase gene which is interrupted, therefore resulting in expression of a dysfunctional protein (see Figure 6). This plasmid has been used to stably transfect HeLa cells and are widely used in biomedical research. When the defective splicing site on the lu-

ciferase pre mRNA is masked with a SSO containing the correct complementary sequence it is eventually restored and enables the induction of a luciferase activity, creating a positive read out.

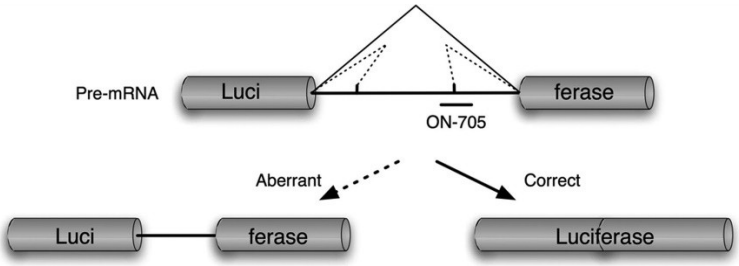

Figure 6. *Schematic illustration of a reporter system for aberrant luciferase pre-mRNA* and the complementary ASO for splice-correction. Retrieved from Guterstam et al. 2008

2.3. Toxicity of cationic polymer-based carriers

The possible non-specific interaction of cationic polymers with polyanions in tissue or cells can cause serious toxicity, especially upon chronic use (*in vivo*). The cationic PEI interacts intensively with negatively charged properties of biological macromolecules or cells, thus causing an early release of the nucleic acid. These interactions are hard to avoid, because they are owed to the inherent structure of polyethylenimines (Juliano 2016). Polyanions that have similar charge properties to nucleic acids like PEI have, bind cationic polymers and lead to early destabilization before the desired target is reached, from which two are representative and ubiquitous in physiological environments, serum albumin and heparin (Wang et al. 2019). These interactions make the e.g., intravenously administration of polyplexes rather challenging. It is important to distinguish between two types of toxicity, firstly cytotoxicity on the cellular level and toxicity on the systemic level (e.g., tissues and organs) and the subsequent side effects in patients.

The molecular mechanisms of the effect on cell viability are highly complex and this thesis only provides a brief insight to this topic. Specific properties like molecular weight and the amount of cationic groups in the polymer are clearly associated with cytotoxicity in various cell lines (Monnery et al. 2017), with higher molecular weight and more cationic properties being more toxic. Two main stages of cytotoxicity are induced by polyplexes, necrosis and initiation of the intrinsic apoptotic pathway.

The destabilization of the plasma membrane results from electrostatic interactions between cationic PEI and negatively charged proteoglycans on the cell surface, which can be determined e.g., by lactat dehydrogenase assay. Disruption of the cell membrane takes place during cellular uptake of the nanoparticle and leads inevitably to necrosis of the cell. Activation of a mitochondrially mediated apoptotic programme occurs as well, resulting from channel formation in the outer mitochondrial membrane due to interactions of cationic moieties of the polymer with the outer mitochondrial membrane. This leads to the release of cytochrome c, subsequent activation of caspase 3, loss of mitochondrial membrane potential, and production of reactive oxygen species (Moghimi et al. 2005; McConnell et al. 2016). The *in vitro* study of Beyerle et al. showed that first contact of the cell with a PEI-based polyplex increases oxidative stress and induced gene expression of antiapoptotic as well as proinflammatory factors. After

24h the expression apoptosis-related genes increased e.g., Bcl2l1, Bax and DNA damage response like Atm and Ercc4. The induction of the intrinsic pathway correlates to the endosomal uptake of PEI, causing rupture of the endosome. The release of its contents generates intracellular stress and can induce apoptosis.

According to the definition by the Nomenclature Committee on Cell Death, a cell is considered dead as soon as “irreversible degeneration of vital cellular functions (notably ATP production and preservation of redox homeostasis) culminating in the loss of cellular integrity (permanent plasma membrane permeabilization or cellular fragmentation)“. Cell death can be divided into two main categories, namely regulated cell death (RCD) or accidental cell death (ACD). RCD is defined as a result of activation of signal transduction modules and therefore can be pharmacologically or genetically altered to some extent, whereas ACD is an uncontrollable form of cell death corresponding to the physical rupture of the cell’s plasma membrane caused by massive physical, chemical, or mechanical influences. There are various types of RCD and each can manifest in necrosis as well as apoptosis of the cell and shows a diverse immunomodulatory profile ranging from anti-inflammatory to pro-inflammatory and immunogenic.(Galluzzi et al. 2018).

After intravenous administration side effects can occur due to the above discussed mechanisms in organs especially in the lung. It could be proven that cationic polymers like LPEI show high affinity to the lung after systemic administration in mice which is probably caused by the small diameter of lung capillaries which are suitable for the transfer of nano-sized polyplexes (Coll et al. 1999; Goula et al. 1998).

2.3.1.Cell models for cytotoxicity

Cells used in biomedical research can be roughly classified into three different groups, namely (i) primary cells, (ii) continuous cell lines, also known as immortalized or indefinite cell lines, and (iii) stem cell lines.

Primary cells are directly derived from tissue or organ using enzymatical or mechanical methods and are slow in growth which results in a limited number of cell generations that can be used for cultivation. Primary cell models have the advantage of closely mimicking in vivo conditions, albeit with a few limitations. They are difficult to obtain, eventually change in culture and exhibit limited life spans. Finally, primary cells undergo aging and senescence, a process where a state of permanent cell cycle arrest in G_0, G_1 or G_2 phase and contact inhibition after forming monolayers in culture. Via subcultures, primary cells can become finite cell lines or continuous cell lines. The latter ones are also called immortalized cell lines which are usually obtained from cancerous cells or transformed cell lines and are characterized by rapid growth to high densities, not contact-inhibited and possibly form multilayers. Stem cells are an undifferentiated or partially differentiated pluripotent cell type. These cells can be cultured to indefinite growth of cells of the same type or alternatively can be induced to produce cells

with specific functions. Hence, they can act as a kind of multipotent precursor for various different cell types (Hernandez-Segura et al. 2018; Verma et al. 2020; Weiskirchen et al. 2023).

Porcine endothelial cells

In this thesis endothelial cells of porcine origin (*Sus scrofa*) were used as a cell model for investigating cytotoxicity of polyplexes. The cells show the characteristic morphology of endothelial phenotype with cobblestone appearance and growth in monolayer, which is best observed with an electron microscope (Burciaga-Nava et al. 2009). One of the challenges is, that the occurrence of fibroblasts hampers the growth of PECs and then the growth of fibroblasts is even accelerated when the PEC monolayer reaches confluency (Golus 2016). Endothelial cells play a crucial role in maintaining vascular function by regulating blood pressure, preventing clotting, and controlling the passage of nutrients, hormones, and leucocytes into and out of the bloodstream as a kind of semipermeable cellular barrier between blood and tissue. They may therefore serve as a potential target for the treatment of such disorders, including gene therapy (Pelisek J, Armeanu S, Nikol S). More important is that the common route of administration for nucleic acid drugs is intravenous injection which can lead to damage of the blood vessels or endothelial layer near or distant to the injection site. Especially endothelial cells are exposed most to relatively high drug concentrations directly after administration, hence potential side-effects like inflammation or thrombosis can occur (Schleger et al. 2004). Additionally, it is well known that swine and humans share similar anatomic and physiologic characteristics e.g., the cardiovascular, urinary, and digestive systems which led to the increased use of swine in preclinical toxicology testing (Swindle et al. 2012). Therefore, it is a suitable cell model for the goal of investigating cytotoxicity of polyplexes.

HeLa cell line

Another model cell line used in this thesis for studying cytotoxicity (and splice correction) is the HeLa cell line. This cell line was originally derived – unfortunately without her consent – from cervical cancer of an African American woman named Henrietta Lacks in the early 1950s. This cell line was the first human cell line established in culture and has become one of the most used in scientific research because it is remarkably prolific and durable and therefore perfectly suitable for studying human cellular and molecular biology (Landry et al. 2013).

There are many different types of HeLa, out of which HeLapLuc705 were mostly used. The pLuc705 in its name results from a reporter plasmid pLuc705 which has been used earlier to achieve stable transfection of HeLa (Nothisen et al. 2018; Riss et al. 2004; Taschauer et al. 2016; Valeur und Berberan-Santos 2011).The plasmid contains the luciferase gene interrupted by a mutated human beta-globin intron 2 (IVS2-705) (Resina et al. 2007) and Hygromycin resistance gene (Thanki et al. 2019). This mutation in the intron causes aberrant splicing of luciferase pre-mRNA, which results in preventing translation of luciferase (Kang et al. 1998) and therefore the expression of a functional protein.

2.3.2.Cell viability assays

The determination of cell viability or cytotoxicity plays a fundamental role in all forms of cell culture and is commonly used in pharmaceutical development. Especially the possibility of testing new compounds in an economic, reproducible, and reliable way aroused great interest in pharmaceutical research, which is enabled by several cell viability methods. The range goes from simple trypan blue dye exclusion assay to complex analysis of individual cells, like RAMAN microscopy and many more methods suitable for specific purposes (Stoddart 2011).

Depending on the aim of an experiment consideration of factors like end-point determination, manual or high-throughput application, type of cells and their metabolic activity, the experimental design and the possible interactions caused by components of the medium or the cell viability reagent is recommended. Furthermore, the effect of the test-substance on the cells, meaning whether it leads to apoptosis or necrosis or has a cytostatic impact should be considered before choosing a specific method for investigating cell viability. Either the number of viable cells or the number of dead cells can be identified using a wide range of different methods. In addition to the classification just mentioned, there are also categorisations based on colorimetric methods, as well as assays that make use of changes in the metabolic environment of the cell. Some of these methods are explained as follows.

The trypan blue exclusion assay determines the number of viable cells present in a cell suspension. It is based on the exclusion of the dye by viable cells with intact plasma membranes, while dead cells are stained, followed by microscopic examination on a haemocytometer (Strober 2001; Riss et al. 2004). Another method to stain dead cells to distinguish them from live cells, is DNA staining with fluorescent DNA binding dyes like DAPI or propidium iodide with subsequent DNA quantification via e.g., fluorescence microscopy or flowcytometry. DAPI and propidium iodide only permeate into dead cells, in contrast to other fluorescent small molecules like Hoechst, which penetrates the cell membrane and intercalates with the DNA, hence is suitable for detecting viable cells, in particular nuclei (Riss et al. 2004). The sulforhodamine B assay is a colorimetric assay used for cell density determination, based on the measurement of cellular protein amount by a pH dependent binding of the dye to cellular proteins. The amount of dye bound is proportional to the retrospectively calculated number of viable cells. This assay is a so called endpoint assay, because fixation of the cells is necessary (Vichai und Kirtikara 2006). There are other colorimetric assays that measure cell viability indirectly via quantification of specific metabolic markers as NADH, caspases, LDH etc. MTT assay, which is an established cell viability assay in biomedical research, uses a purple tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that gets reduced to yellow formazan, which correlates with cell viability and cell proliferation. Reduction of MTT is accomplished by NADPH, FADH, FMNH, and NADH, but not by cytochromes. The main disadvantage of this assay is that tetrazolium salts in general are not water-soluble, therefore need to be solubilized by e.g., DMSO or another suitable organic solvent, which is highly cytotoxic and make time-course experiments and multiplexing with other cell-based *in vitro* assays impossible (Rampersad 2012). Another way to detect dead cells is the lactate dehydrogenase-assay (LDH) because damaged cells with membrane rupture excrete cytosolic compounds like the enzyme LDH. It catalyses the conversion of pyruvate to lactate and in this process reduces NAD to NADH. The reduction potential of NADH enables the reduction of various substrates

to coloured, fluorescent or luminogenic molecules which can be detected with an appropriate method (Riss et al. 2004). ATP can be quantified as well, because it correlates with cell viability and energy of the cell, but the ATP assay in contrast is not a colorimetric method like the above-mentioned assays. When membrane integrity is lost, cells lose the ability to produce ATP and endogenous ATPases quickly depletes remaining ATP from the cytoplasm. To measure the amount of ATP in cytoplasm, luciferase is of great use. In presence of ATP, luciferase converts luciferin into light which can be determined by a luminometer, hence light intensity correlates with cell viability (Riss et al. 2004). One of the most frequently used colorimetric assays for the determination of cell proliferation compared to other methods presented above is the resazurin or Alamar Blue assay, which will be discussed in 2.3.2. One potential drawback of some of these metabolic assays is there is no differentiation between cells that are actively dividing and those that are quiescent which may result in an over-estimation of cell number (Rampersad 2012).

2.3.3.Resazurin assay

Resazurin also known as Alamar Blue (IUPAC: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is a redox indicator used for determination of cell viability by monitoring the reducing environment of viable cells. It was first used in 1929 for assessing bacterial or yeast contamination in milk by Pesch and Simmert. It is water-soluble, stable in cell culture medium, non-toxic and membrane permeable (despite the negative charge at physiological pH) which gives the possibility of multiplexing this assay and enables continuous monitoring of the cells. Blue non-fluorescent Resazurin is reduced to pink and highly fluorescent resorufin, which is proportional to the live cell number, by NADH, NADPH, FADH or other reductive species in the presence of mitochondrial or cytoplasmic reductases (see Figure 7). There are mitochondrial reductases like cytochromes and cytosolic ones like diaphorase, oxidoreductase and flavin reductase which all have the ability to reduce resazurin. As the dye accepts electrons, it changes from the oxidized, non-fluorescent blue state to the reduced, fluorescent pink state. The resazurin reduction may signify an impairment of cellular metabolism but is not necessarily specific to inhibition of electron transport and mitochondrial dysfunction (Rampersad 2012; Lavogina et al. 2022). The reaction from resazurin to resorufin is the primary reduction reaction, which is irreversible. Resorufin can be (reversibly) reduced in a secondary reaction into two distinct degradation products, dependent on the pH of the medium or solution. At pH > 4 resorufin is degraded to protonated resorufin anion and at pH < 4 it is converted to colourless dihydroresorufin. The reduction of resorufin is not only dependent on the pH, but on the presence of O_2, as well as reducing agents like NADH and the intrinsic reducing capacity of the cell line used. Furthermore, the initial cell seeding density is from importance, because confluency of the well surface increases with time passed between seeding and (Vieira-da-Silva und Castanho 2023; Zhao et al. 2011; Lavogina et al. 2022).

The quantification of the number of viable cells can be determined via fluorescence or absorbance read-out however, the fluorometric determination is superior in terms of sensitivity. In fluorescence, photon emission is based on energy transitions from excited states to ground states. This energy is gained through absorption of (UV-) light and results in the emission of cold light with higher wavelength than the exciting light and ends simultaneously with the end of excitation. In contrast to bioluminescence, fluorescence is much brighter, because photons generating the excited stage can be introduced into samples very effectively. A disadvantage of the high photon influx is that it can lead to very high background signals, which may influence the sensitivity of the assay. It can also be affected by the presence of interfering fluorophores within the biological samples e.g., phenol red in cell culture medium, whereas bioluminescence is less hindered (Fan und Wood 2007; Valeur und Berberan-Santos 2011; Al-Nasiry et al. 2007).

The reduction of resazurin to resorufin at physiological pH results in a shift in absorbance spectrum (resazurin: λ_{max} = 600 nm, resorufin: λ_{max} = 570 nm). Additionally, fluorescence can be registered when resorufin is excited with wavelengths corresponding to its absorbance maximum (Lavogina et al. 2022). Fluorometric readouts using resorufin have several advantages, such as long excitation and emission wavelength (572/585 nm), high fluorescence quantum yield, favourable stability to light irradiation and pH changes (pH 6.0– 10.0), high water solubility and biocompatibility (Tian et al. 2021).

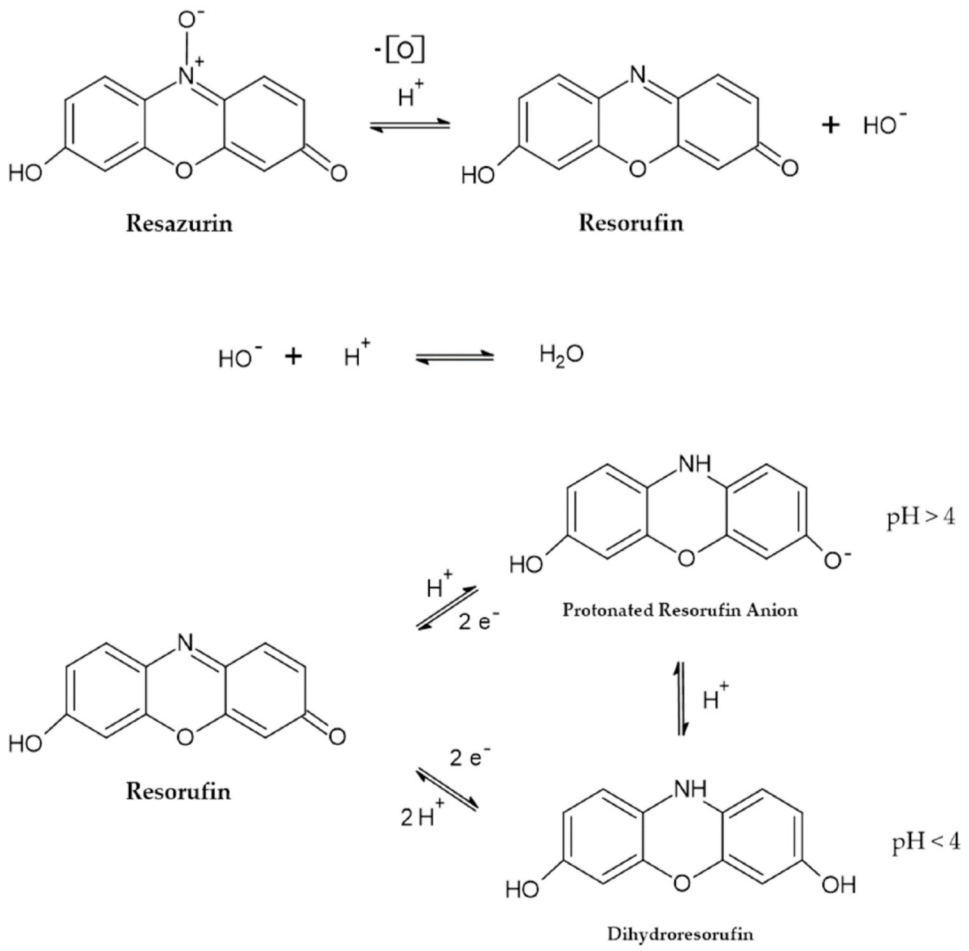

Figure 7. *Reaction scheme of resazurin redox and acid-base reaction into resorufin.*
Adapted from Vieira-da-Silva und Castanho.

3. Materials and Methods

3.1. List of used Equipment

Biological Safety cabinet, Thermo Fisher Scientific	Pipette tips, Nerbe plus GmbH
Centrifuge tubes 15ml (CT-15), Starlab	Plate reader, Infinite M200 Pro, Tecan
Centrifuge tubes 50ml (CT-50), Starlab	Plate shaker (Eppendorf)
Centrifuge, Thermo Fisher Scientific	Reagent reservoirs, VWR International
Counting chamber, Neubauer-improved, Paul Marienfeld GmbH & Co. KG	Refrigerator(4°C), Allectric
Eppendorf tubes, Nerbe plus GmbH	Serological pipettes 5ml, 10ml, 25ml, Sarstedt
EppendorfR ResearchR plus pipettes, Sigma-Aldrich	Sterile filters, cellulose acetate, 0,22 µm, VWR International
Freezer (-20°C, -80°C), Thermo Scientific	Syringes, BD Discardit
Incubator (37°C, 5% CO2), Thermo Fisher Scientific	TC flask T75, standard, vent. cap., Sarstedt
Inverted Microscope, Motic	Transparent 96-well flat-bottom microplate, sterile (Greiner)
Luminometer GloMax® Navigator, Promega	Vacuum aspiration system, Integra Biosciences
Mr. Frosty Freezing Container, Thermo Scientific	Vortex mixer, Velp Scientifica
Needles, 100 Sterican, Braun	Water bath, Memmert GmbH
Pipette boy, VWR International	White 96-well flat-bottom microplate, transparent bottom, sterile (Greiner)

3.2. List of reagents

Amphotericin B solution, Sigma Aldrich	HEPES buffered glucose (HBG, 5 % glucose w/v, 20 mM HEPES pH 7.4), MMCT (University of Vienna)
Antisense Oligonucleotides, GlaxoSmithKline (GSK, UK) LucSSO: 5'-CCUCUUACCUCAGUUACA-3' (MW = 6472,61 Da) NegSSO 5'-CUUGUUAUACCACUUACA-3' (MW = 6496,64 Da)	Hygromycin B solution, Sigma-Aldrich L-Glutamine 200mM, Sigma-Aldrich Linear polyethylenimine (LPEI), 3kDa, MMCT (University of Vienna)
BCA Protein Assay Kit, Thermo Scientific	Lipofectamine 3000 transfection reagent, Thermo Fisher
Branched polyethylenimine (BPEI), MMCT (University of Vienna)	Luciferase assay reagent buffer + luciferin (LBL), MMCT (University of Vienna)
HeLa wt (CCL2, ATCC, Manassas, USA) HeLa pLuc705 (obtained within the IMI collaborative project COMPACT) PEC (prepared by Julia Golus from porcine aorta tissue from Fleischerei Hödl)	Medium199, Sigma-Aldrich MilliQ-water, Sartorius Nanodrop, GE
Crosslinked polyethylenimine (C-LPEI HB, 0.5 and 1.0), MMCT (University of Vienna)	Passive Lysis Buffer 5x, Promega
Dimethyl sulfoxide (DMSO), Sigma-Aldrich	Penicillin-Streptomycin, Sigma-Aldrich
Dulbecco's modified eagle's medium (DMEM) high glucose, Sigma-Aldrich	Resazurin Alamar blue HS, Thermo Fisher Scientific
Dulbecco's phosphate buffered saline (DPBS), Sigma-Aldrich	Resazurin, Acros Organics
Fetal bovine serum (FBS), Sigma-Aldrich	Resazurin assay kit, VWR
Geneticin (G418 solution), Gibco	TrypLE Express 1X, Gibco

3.3. Cell culture and seeding

3.3.1. Preparation of complete medium

Specific cell lines, such as HeLa or PEC need appropriate medium to ensure their growth and health, in order to use them for experiments. In these experiments two different commercial growth media were used, the preparation for both is explained in the next passages.

Dulbecco's modified eagle's medium (DMEM) high glucose for HeLa

The preparation of complete medium was always carried out in a biological safety cabinet using serological pipettes. DMEM high glucose medium (500 ml bottle), referred to as basal medium here, is stored at 4°C and has to be taken into the biosafety cabinet before opened. First, 50 ml of this basal medium must be transferred into a CT-50 and set aside. After this, 5 ml of Penicillin-Streptomycin, 10 ml of Glutamine and 50 ml FBS must be added to the bottle. The final concentrations should be 1% of Penicillin-Streptomycin, 2% of Glutamine and 10% of FBS. In the end, make sure the medium is mixed well.
HeLapLuc705 need an additional antibiotic for selection pressure. 50 ml aliquots of complete medium are stored in a CT-50, where 200 µl of Hygromycin solution are added. The final concentration of Hygromycin in medium should be 0,04%.

Medium 199 for porcine endothelial cells

The workflow is the same as shown above, only the ingredients and concentrations are different. After opening the basal medium, 100 ml must be transferred in to two CT-50. Then 100 ml of FBS, 25 ml of Penicillin-Streptomycin and 5 ml of Amphotericin B (conc. 2,5 mg/ml) are added. The final concentrations should be 5% of Penicillin-Streptomycin, 20% FBS and 1% Amphotericin B. Also, here it must be assured that the medium is mixed well at the end.

3.3.2. Cell culture: HeLa wt and HeLa pLuc705

HeLa cells were kept in T75-flasks in the incubator at 37°C and 5% CO_2. Before splitting and seeding, turbidity and colour of the medium were checked visually, while the confluency was examined with a microscope. A confluency of 80-90% is desired. The time until cells are confluent, varies from cell line to cell line, in this case a splitting ratio of 1:10 (Monday to Friday) and 1:5 (Friday to Monday).
After taking the flask out of the incubator, it was transferred into the biological safety cabinet, where the medium was removed entirely with a serological pipette and discarded. To remove any remaining medium and debris, PBS was used to wash the cells. The next step is to detach the cell layer from the bottom of the flask with 1-2 ml of Trypsin, which was diluted with 8-9 ml medium after a short incubation to stop the enzymatic reaction. This cell solution was transferred to a CT15 and after 5 minutes of centrifugation at 200xg at room temperature, a pellet can be received on the bottom of the tube. The

pellet was resuspended in 1 ml of medium and a specific volume, depending on the desired splitting ratio, of this solution was added to the medium in a T75.

For optimizing the Resazurin assay, HeLa pLuc705 were seeded in different cell numbers: 5000, 10000, 15000, 20000, 25000 and 30000 cells/well as shown in Figure 8. The calculations for seeding were done with Microsoft Excel. After seeding, the plate was kept in the incubator for 24 hours.
For multiplexing experiments, the cells were seeded in triplicates in a white 96 well plate, the end-concentration per well is 10000 cells/well.

3.3.3. Cell culture: Porcine endothelial cells

Freshly thawed porcine endothelial cells were first kept in T25-flasks and transferred into T75-flasks after reaching the desired confluency of 80%. They were always kept in the incubator at 37°C and 5% CO_2. A medium change was done once week. Before splitting and seeding, turbidity and colour of the medium were checked visually, while the confluency was examined with a microscope. The splitting ratio is low and ranges between 1:2 and 1:4 every four days with medium exchange between the days where the cells get split.

After taking the flask out of the incubator, the procedure is the same as already mentioned before, until the detaching process. To detach the cell layer from the bottom of the flask 1 ml of TrypLE is used, which was diluted with 3 ml medium after a short incubation of 4 minutes in the incubator to stop the enzymatic reaction. This cell solution was transferred to a T75-flask in aliquots of 1 ml per flask with a final volume of 10 ml.

For optimizing the Resazurin assay, PEC were seeded in different cell numbers: 5000, 10000, 15000, 20000, 25000 and 30000 cells/well and wells containing only medium for the background, as shown in Figure 8. The calculations for seeding were done with Microsoft Excel. After seeding, the plate was kept in the incubator overnight.

For cell viability experiments after polyplex treatment, the PEC were seeded in triplicates in a transparent 96 well plate, the final concentration per well is 10000 cells/well.

3.4. Optimization of cell viability assays

For the cell viability assay 110 µl medium is removed from each well to add 10 µl of housemade Resazurin solution (preparation of it is explained in the next section) or the commercial equivalents VWR Resazurin and Alamar Blue HS. The following procedure is the same for all reagents. After an incubation time of 1 hour, the supernatant was used to read the relative fluorescence units (RFU) with the Tecan plate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. This procedure for the Resazurin assay is the same for experiments with HeLa as well as experiments with PEC.

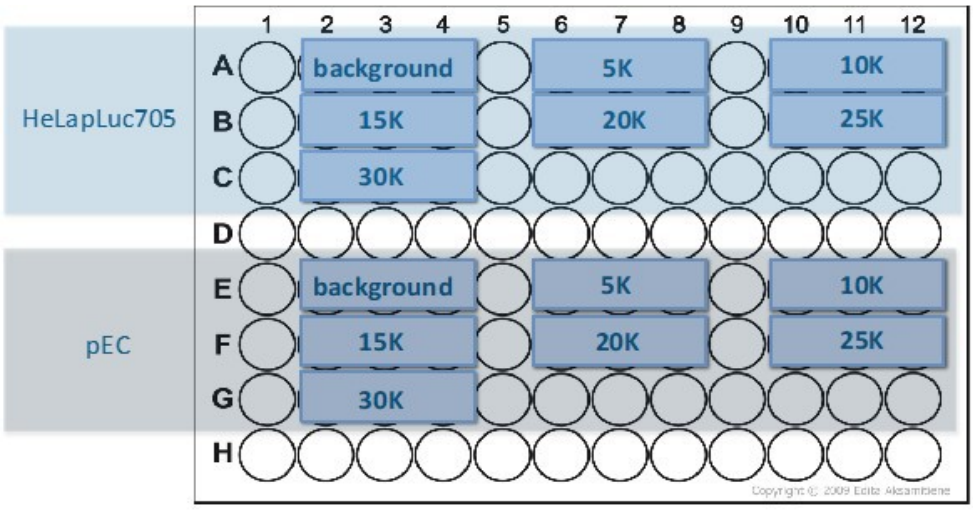

Figure 8. *Schematic overview of 96 well plate for the optimization of cell viability assays.*
Cell numbers of HeLa pLuc075 and PEC were seeded in triplicates.

3.4.1. Preparation of housemade resazurin solution

The desired final concentration is 0,1 mg Resazurin Natrium Salt/ml Phosphate Buffered Saline and must be prepared 1 hour in advance to obtain a stable solution and subsequently a stabile fluorescence signal. 1,6 mg of Resazurin Natrium salt was weighed and dissolved in 1,6 ml PBS. To achieve 0,1 mg/ml, a 200 µl aliquot of the solution was diluted (ratio of 1:10) with 1800 µl PBS. Finally, the Resazurin solution is sterile filtered using a syringe and a sterile filter (0,22 µm pore-diameter). For 96 well plates, 10 µl Resazurin solution were added to 90 µl medium in each well.

3.4.1. Preparation of commercial Resazurin kits

The commercially available products VWR Resazurin and Alamar Blue HS did not need preparation and were used as recommended in the manufacturer's protocol. For 96 well plates, 10 µl Resazurin solution were added to 90 µl medium in each well.

3.5. Characterization of basal firefly Signal in HeLa pLuc705

To investigate the firefly luciferase expression in HeLa pLuc705 a BCA-/firefly assay was carried out for characterising the basal luciferase signal, with HeLa wt as control. The assay was done following the protocol below of Daniela Gruber (2021, University of Vienna).

At first PLB2X is prepared out of 200 µl PLB5X and 300 µl MilliQ water, as well as the BSA-Standards. After an incubation time of 20 min at room temperature, the PLB can be added to the BSA-Standards. The final volume of each Sample is 100 µl/Eppendorf tube (see table below).

Sample ID	MilliQ water [µL]	BSA 2mg/mL [µL]	PLB2X [µL]	BSA [µg]
8A	-	50,00	50,00	20
7B	10,00	40,00	50,00	16
6C	20,00	30,00	50,00	12
5D	30,00	20,00	50,00	8
4E	40,00	10,00	50,00	4
3F	45,00	5,00	50,00	2
2G	50,00	-	50,00	-

Figure 9. *Modified BSA dilutions protocol (Gruber D, 2021)*

20 µl of the above BSA-dilutions, starting with the lowest concentration 2G (“blank”), are transferred in triplicates into a transparent 96 well plate to obtain a calibration line. The working-reagent must be prepared from the BCA-Protein-assay kit under exclusion of light in a laminar flow and must also be protected from light during the incubation period of 20 min until use. From this reagent 200 µl are transferred to each well with a multichannel pipette, shaken for 30 seconds at RPM 300 and room temperature and afterwards put in the incubator at 37°C for 30 minutes. During this time a change of colour can be observed, depending on the protein concentration. Also, the plate must be kept under light-protection until reading. For reading, Tecan plate reader was used to measure the absorption at 562 nm (no. of flashes 25, bandwidth 9nm). A standard curve with a coefficient of determination of 0,99 was desired and was created with Microsoft Excel.

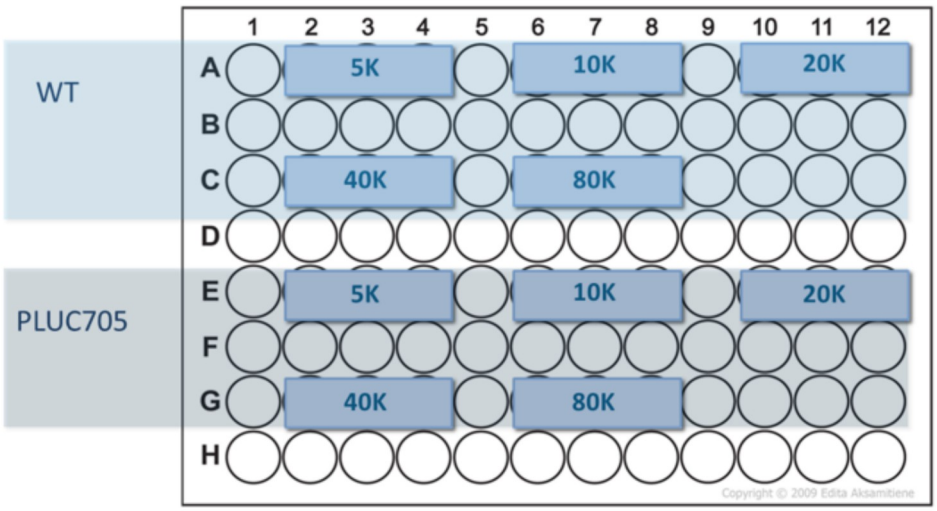

Figure 10. *Schematic overview of a 96 well plate showing cell numbers per well of HeLa wt (control) and HeLa pLuc705 for firefly luciferase assay.*

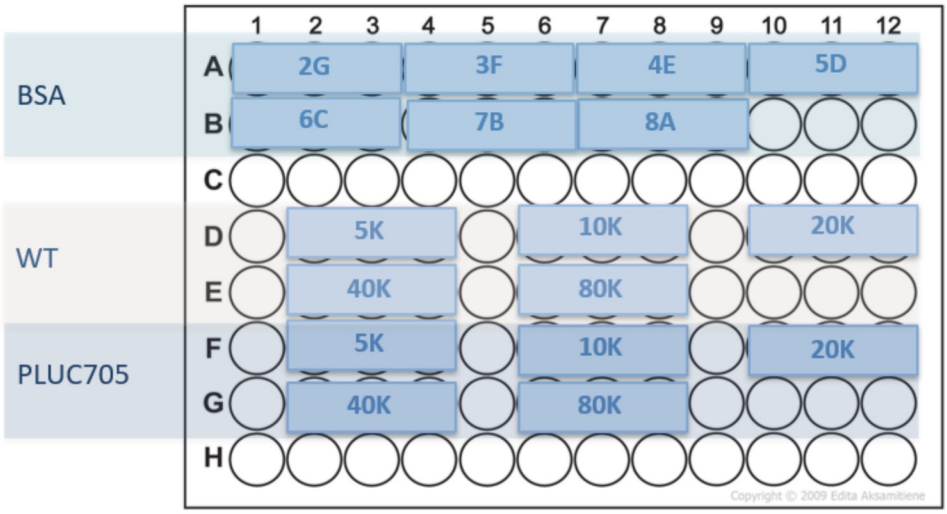

Figure 11. *Schematic overview of a 96 well plate showing the BSA Standards.*
See Figure 9. Modified BSA dilutions protocol (Gruber D, 2021) and cell numbers per well of HeLa wt (control) and HeLa pLuc705 for BCA/firefly assay.

For examining the luciferase expression, the white plate which was seeded the day before, has to be processed as already mentioned until the process of scratching. Here, 20µl of the lysate of each well is transferred in triplicates to the transparent plate. The remaining 10 µl in the white plate are used to be read with the Luminometer. The standardized reading order was to read HeLa wt first and afterwards HeLa pLuc705. The aim is to obtain the normalised values for RLU/1 µg from the RLU/well which was done with the equations below in Microsoft Excel:

Calculation of the protein concentration via BCA-assay:

$$Absorption - blank = protein\ amount\ in\ 20\ \mu l\ (per\ well)$$

$$protein\ amount\ in\ 20\ \mu l \times \frac{30}{20} = protein\ amount\ in\ 30\ \mu l$$

Calculation of the RLU/1 µg protein via firefly luciferase assay:

$$RLU\ in\ 10\ \mu l\ (per\ well) \times \frac{30}{10} = RLU\ in\ 30\ \mu l$$

$$\frac{RLU}{1\ \mu g\ protein} = \frac{RLU\ in\ 30\ \mu l}{protein\ amount\ in\ 30\ \mu l}$$

3.6. Multiplexing: combining splice correction readout with cytotoxicity assay

Figure 12. *General workflow for Multiplexing*
(Day 1) Cells were seeded in 96 well plates, (Day 2) Polyplexes were created and added to the wells, followed by 24 hours of incubation, (Day 3) firefly and resazurin assay reading

3.6.1. Generating Polyplexes and *in vitro* delivery

The Polyplexes in this study are polymer-based nanoparticles that consist of a cationic polymer combined with an antisense oligonucleotide, so called “splice-switching oligo”. These two components are oppositely charged thus, they build complexes, which can be used for transfecting cells. The sequence and molecular weight of both types of ASO are found in section 3.2.

HEPES buffered glucose is utilised as a buffer for the polyplexes and is best used at room temperature. For efficient transfection, basal medium has to be prepared and warmed in the water bath. The reason for this procedure is that HeLa cells show better transfection efficiency when serum-free basal medium is used, therefore the absorption of the nanoparticle by the cell is increased (Chong et al. 2021). Polyplexes were always generated in advance, during the incubation time of the 96 well plate.
Another important step of procedure to do in advance is the quantification of the RNA, which is done with the device Nanodrop. The concentration that are measured are needed for calculating the concentrations used for polyplexing.

Two types of ASO were used: LucSSO for splice-correction and NegSSO as a negative control. Both were used to achieve two different concentrations of polyplexes: 160 pmol/well and 80 pmol/well with an N/P ratio of 9. ASOs are kept at -80°C in MQ-water and diluted with HEPES buffered glucose to create working stocks in the desired concentration. The polymer batches used were C-LPEI 0.5 (where the molar ratio of LPEI (1 kDa) and the homo-bifunctional crosslinker DSP ((dithiobis(succinimidyl propionate)), also known as Lomant’s reagent, was 1:0.5 during the crosslinking reaction), C-LPEI 1.0 (molar ratio LPEI:DSP 1:1) and a batch of C-LPEI, which has been stored for 4 years at -80°C (C-LPEI HB) (Breunig et al. 2007). In addition, LPEI (3k Da) (non-crosslinked control), Lipofectamine 3000 (positive control) and branched PEI (25kDa), BPEI (positive control) (Bonner et al. 2013). The concentrations of the polymers were known and used for the calculation for the needed amount to get 160 pmol ASO/well (conc. A) and/or 80 pmol ASO/well (conc. B). For 80 pmol/well BPEI and Lipofectamine were not used, and their effect therefore not examined.

To create the working stocks of each polymer and ASO, HBG is transferred to Eppendorf tubes. The volume for the PEI-stocks is 31,85 µl HBG and for the ASO stocks 36 µl HBG/Eppendorf tube. Then 28,15 µl PEI stock (conc. = 1mg/ml in water) and 24,00 µl ASO (conc. = 1mg/ml in water) stock are

added into the corresponding tube, to achieve a final volume of 60 µl PEI in HBG and ASO in HBG. The polymer solution must be vortexed and spun down before usage. In order not to damage the very fragile ASO through shearing forces, it is mixed by tapping the Eppendorf tube and carefully spun down. For generating the polyplexes the technique of flash-pipetting must be used. The polymer is transferred to the ASO with a single pipet, using filter tips. It is crucial to let out the remaining air in the pipet tip, before mixing, plus it should be avoided to have bubbles in the solution. After letting all the air out, the pipet tip is moved into the solution of ASO and HBG and immediately the solution must be pipetted up and down, at least 20 times in a very fast way.

Polyplexes with Lipofectamine 3000 were created according to the manufacturer's protocol with the following adaptions:
For 100 ng ASO per well 1,0 µl of P3000 reagent and 25 µl Opti-MEM are needed to create the ASO-lipid-complex. 1,5 µl Lipofectamine 3000 reagent and 25 µl Opti-MEM are mixed and the ASO-lipid complex is added to obtain a final volume of 50 µl Polyplex solution. After 10 minutes of incubation 10 µl of the solution is added to each well.

The plate which was seeded 24 hours ago, was taken out of the incubator and checked visually with the microscope. Then all the medium in the wells was aspired entirely with a vacuum pump and exchanged with 76 µl (for conc. A) and 88 µl (for conc. B) basal medium and after this step, the prepared polyplexes are added to each well (except for the control). The volume of polyplex solution for conc. A is 24 µl/well and 12 µl/well, resulting in a final volume of 100 µl/well (see Figure 13). After 4 hours of incubation 100 µl complete medium was added to each well. Multiplexing was done 24h hours later (see 3.6).

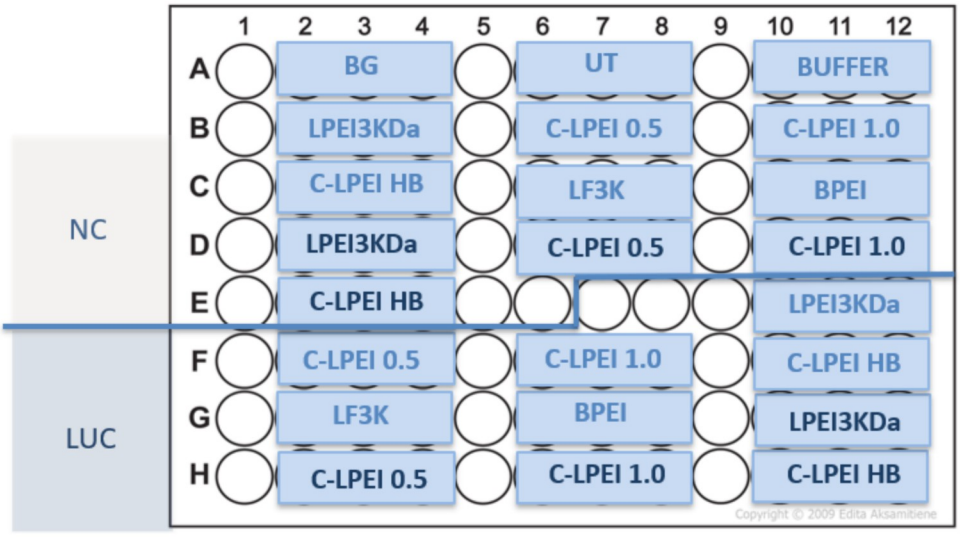

Figure 13. *Schematic overview of a 96 well plate for treatments used in Multiplexing assay with HeLa pLuc075.* Triplicates for background (BG), untreated cells (UT) and buffer-only treated cells were seeded, as well as triplicates for each polymer in different concentrations shown in different shaded colours: (light blue) Polyplex concentration (A) of 160 pmol SSO/well, (dark blue) Polyplex concentration (B) of 80 pmol SSO/well.

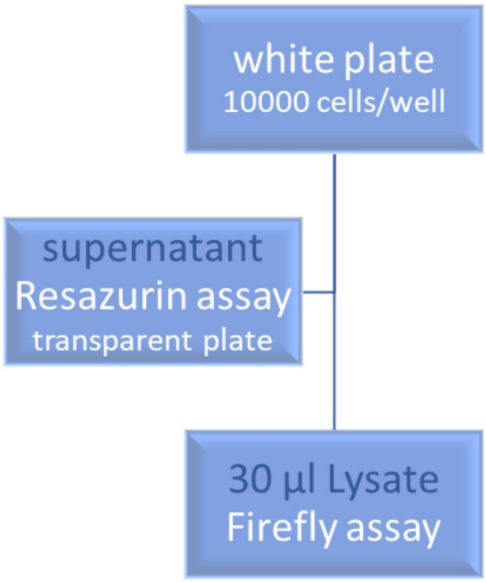

Figure 14. *Detailed workflow for Multiplexing*
In a white 96 well plate HeLa pLuc705 were seeded as explained in 3.3.2. The supernatant was used to investigate cell viability via Resazurin assay, the remaining cells were lysed and the resulting lysate was subsequently used for examining firefly luciferase activity

3.6.2. Firefly assay for investigating splice-correction

HeLapLuc705 in the wells of the white plate are washed with 200 µl PBS using a multichannel pipette. For cell lysis 30 µl of passive lysis buffer 1x (PLB1x), which must be premade out of PLB5x and MilliQ water (MQ) and was then pipetted into each well with a single channel pipette. The plate is incubated on the shaker at room temperature at 500 rpm. Each well containing cells must be scratched very carefully, best using a pipette tip.
The read out is done with a Luminometer, which injects 10 µl firefly Substrate automatically in each well and measures relative light units (RLU) per 30 µl. The aim was to examine the fold increase in splice correction, the following equation was used:

$$\frac{\text{RLU LucSSO}}{\text{RLU NegSSO}} = \text{fold increase in splice correction}$$

3.6.3. Resazurin assay for investigating cytotoxicity of polyplexes

To examine the viability of HeLa pLuc705 and PEC after polyplex treatment, the cells were seeded as shown in Figure 13 (for HeLa only). 110 µl medium is removed from each well to add 10 µl resazurin (Alamar Blue HS), as recommended in the manufacturer's protocol. After an incubation time of 1 hour, the whole supernatant of each well is transferred into a transparent plate to read the relative fluorescence units (RFU) with the Tecan plate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

This procedure for the Resazurin assay is the same for experiments with HeLa pLuc705, as well as experiments with PEC. Buffer-treated cells were compared to SSO treated cells, calculated in %, whereas buffer-treated cells' cell viability was set to 100%.

4. Results and discussion

4.1. Optimization of cell viability assays

There are numerous possibilities in life sciences to analyse cytotoxicity or changes in cell viability after treatment with specific substances or molecules. In this thesis, both the Resazurin assay and the MTT assay were optimised, but subsequently only the Resazurin method was used. The data for MTT can be found in the appendix. Investigating this subject was from importance, because studies have already shown that polyplexes, especially some polymers used for complexation, have cytotoxic potential (Wightman et al. 2001).

For optimizing this assay HeLa pLuc705 and PEC were used to determine the cell viability without any treatment. Cells were seeded in different cell numbers (5000 – 30000 cells/well) in 96 well plates, followed by adding Resazurin solution after 24 hours incubation. Fluorescence reading was done with a plate reader, using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Data about the incubation duration of 4h (but subsequently used incubation of 1h) of the resazurin solution can be found in the appendix (see 8.3.1).

4.1.1. Stability of housemade resazurin

The aim was to investigate whether utilising a housemade Resazurin solution shows any variation in linearity or sensitivity if prepared freshly or prepared in advance since a precise, as well as a time- and cost-effective method is pivotal for achieving reproducible and consistent outcomes in a thesis or study. A calibration curve of resazurin was prepared to provide a method of determining cell viability for further experiments, where relative fluorescence units are plotted against the number of cells/well as demonstrated in Figure 8.

Independently from the reading mode, it is evident that the relative fluorescence units become stagnant in correlation with the age of the buffer used. The RFU values range from five thousand to thirty thousand using the homemade buffer, whereas the values of the five-days-old solution range from one thousand to eight thousand, and the values of the twelve-days-old one even ranges from only one thousand to six thousand, which clearly demonstrates a decrease in stability. It is striking that the R-squares of each experiment differ from each other. One of the reasons simply could be a loss of the buffer's sensitivity, especially in case of the five-days-old Resazurin (see Figure 15), which becomes stagnant after more than five days. Another reason may be the increasing age of the solution which was used under most possible light-protection, but taking the buffer out of the fridge several times may leads to early reduction of resazurin to resorufin (Bueno et al. 2002). Also, the cell density could have played a role, because the results for fresh resazurin are more variant in higher cell numbers (beginning at twenty-thousand cells/well).

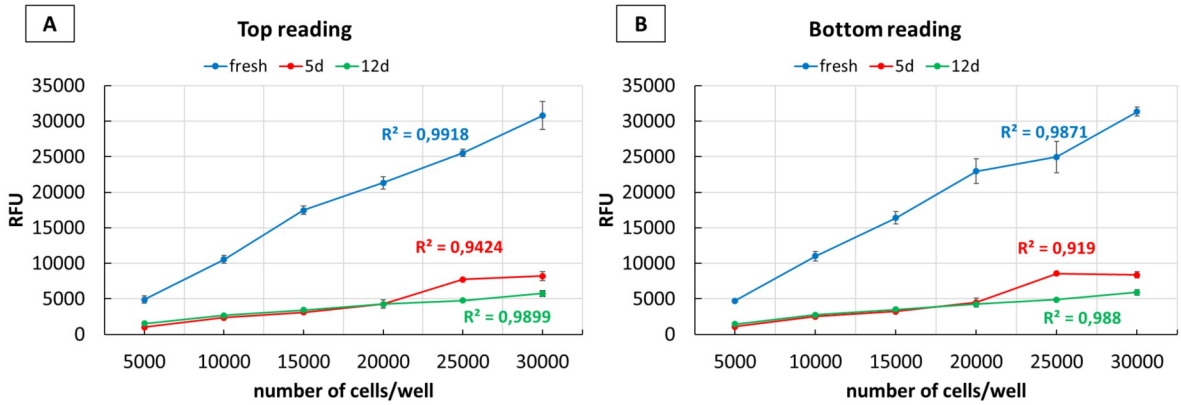

Figure 15. *Resazurin calibration curves with relative fluorescence units (RFU) plotted against number of HeLapLuc705 seeded.*

Comparison of cell viability readout from freshly prepared (blue curve), five-days-old (red curve) and twelve-days-old (green curve) resazurin solution when read in two different reading modes (A) top and (B) bottom reading mode. HeLa pLuc705 cells were seeded in above mentioned cell densities in triplicates, followed by 24 hours of incubation and desired resazurin treatment with an incubation duration of 1 hour. Each graph shows the mean ± standard deviation of n=1 experiment.

4.1.2.Housemade Resazurin vs. commercial Resazurin Assay Kits

The objective was to examine the differences between a housemade Resazurin solution and a commercial product (Alamar Blue HS), concerning linear response to different cell numbers of HeLa pLuc705 and sensitivity of the assay. The corresponding data for porcine endothelial cells is to be found in the appendix (see 8.3.2).

Both reagents showed no difference in linearity as shown in Figure 16, however, it is noteworthy that the RFU values differ, indicating that the commercial product is more sensitive than the in-house Resazurin solution. Also, the Mann-Whitney-U-test which was performed retrospectively shows that Alamar Blue HS produces significantly higher fluorescence signals than the freshly prepared solution with resazurin powder (see Figure 17).

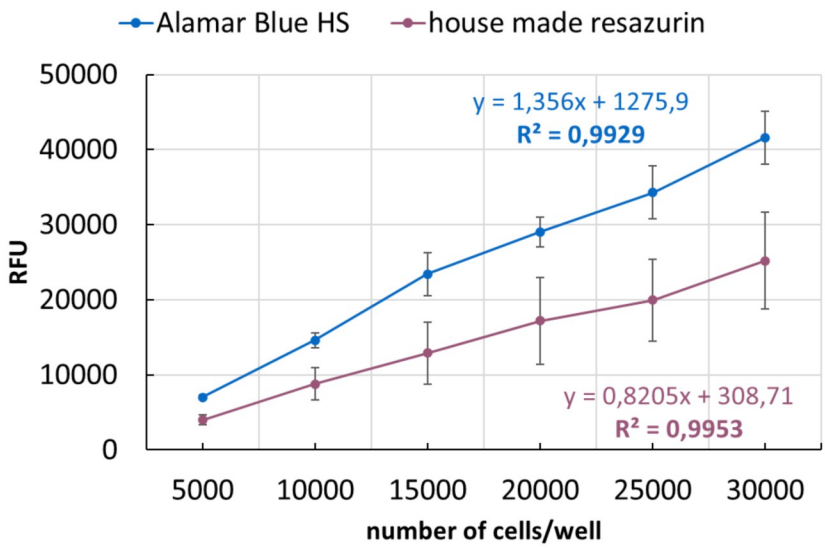

Figure 16. *Comparison of Alamar Blue HS (blue) and housemade Resazurin (violet) in HeLa pLuc705* with relative fluorescence units (RFU) plotted against number of cells (per well*)*. HeLa pLuc705 were seeded in above mentioned cell densities in triplicates, followed by 24 hours of incubation and resazurin treatment with an incubation duration of 1 hour. The graph shows the mean ± standard deviation of n=3 experiments.

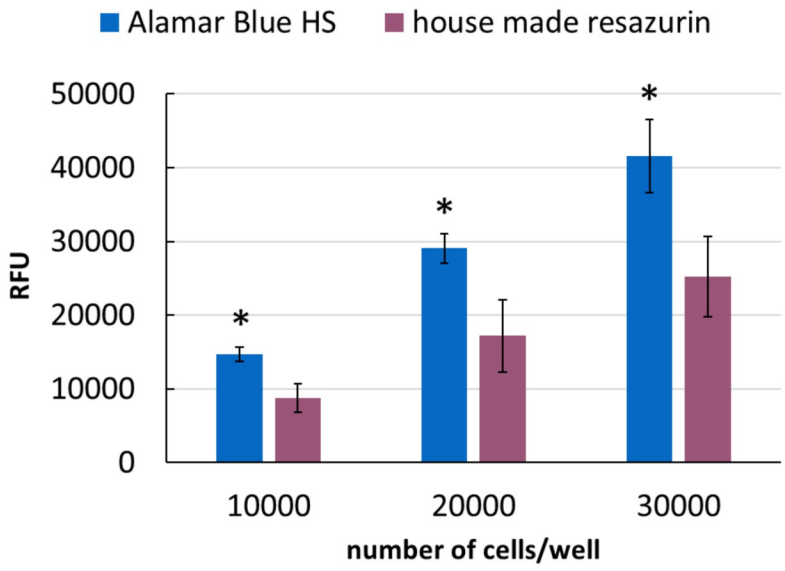

Figure 17. *Mann-Whitney-U-test of using Alamar Blue HS vs. housemade resazurin.* *P<0.05 and P<0.01. The data show the mean ± standard deviation of n=3 experiments

The commercial product is pricier but easier to handle as it is ready to use without the need for preparation time. In contrast, preparing a fresh solution from resazurin sodium salt and PBS is more cost-effective but requires greater time investment and risks for errors, thus rendering it less appealing for research studies. This apparent disadvantage can be avoided by establishing a standardised protocols for the assay like Lavogina et al. showed in their studies. One of the key points in establishing such a protocol is to standardise it for each cell line separately, as well as the dynamic range, because the size of the cells and its cellular metabolism affecting the reaction to resorufin, differ in many cell lines.

4.1.3.Top- vs. Bottom-reading

Furthermore, two different types of reading were examined, namely “top-“ and “bottom-reading”, which means that the wells are read either from above or below. The investigation concerns the assay using the housemade resazurin and two distinct commercial products, namely Alamar Blue HS and VWR Resazurin. The difference in RFUs between the two reading modes is hardly present in commercial products as shown in Figure 18 and Figure 19. One experiment with VWR Resazurin (Figure 18) and Alamar Blue HS (Figure 19) was carried out, producing contradictory results. The RFU for wells treated with VWR Resazurin is slightly increased when read from the bottom, while the RFU of Alamar Blue HS tend to decrease when bottom-reading was used.

In conclusion, there is no remarkable difference between the two modes of reading. In this thesis, bottom-reading was subsequently used for Multiplexing and Resazurin experiments, like recommended in the manufacturer’s protocol of Alamar Blue HS.

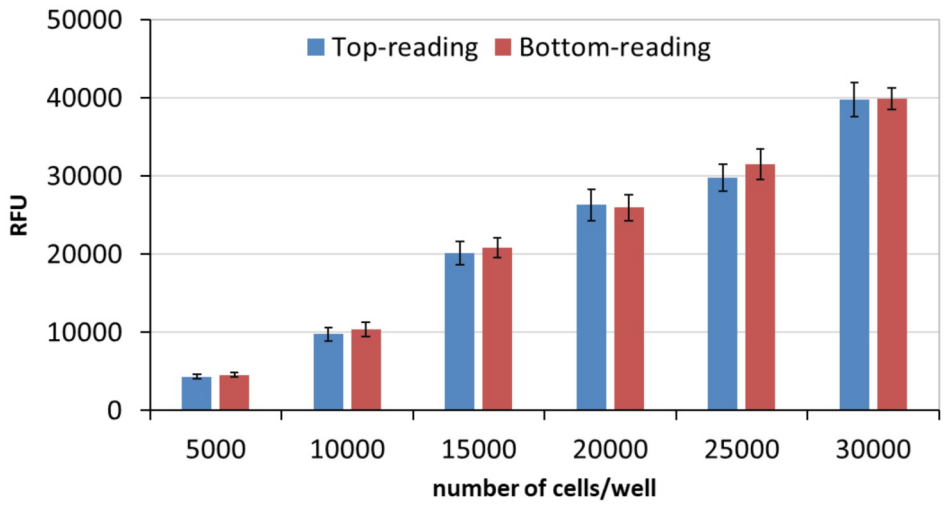

Figure 18. *Comparison of top- and bottom reading using* ***VWR Resazurin***. HeLa pLuc705 were seeded in above mentioned cell densities in triplicates. Relative fluorescence units are plotted against number of cells/well. The graph shows the mean ± standard deviation of n=1 experiment.

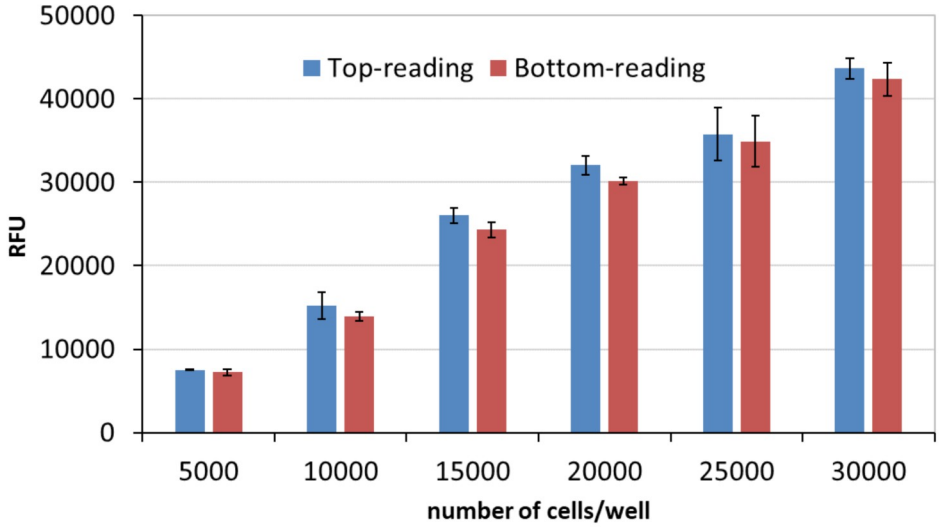

Figure 19. *Comparison of top- and bottom reading using* ***Alamar Blue HS***. HeLa pLuc705 were seeded in above mentioned cell densities in triplicates. Relative fluorescence units are plotted against number of cells/well. The graph shows the mean ± standard deviation of n=1 experiment.

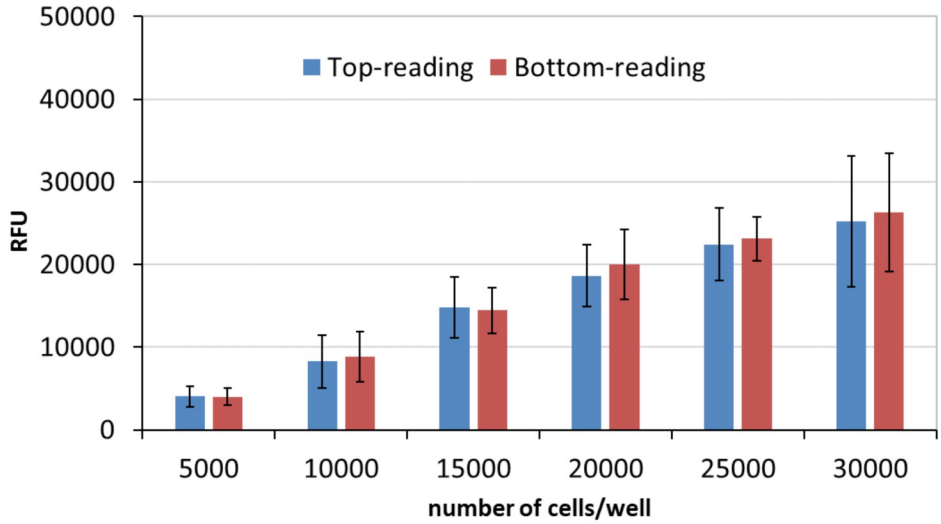

Figure 20. *Comparison of top- and bottom reading using* ***housemade Resazurin***. HeLa pLuc705 were seeded in above mentioned cell densities in triplicates. Relative fluorescence units are plotted against number of cells/well. The graph shows the mean ± standard deviation of n=2 experiments.

4.2. Cytotoxicity of polyplexes in PEC

A commercial Resazurin assay kit (Alamar Blue HS) was used to examine cell viability of negative SSO-treated porcine endothelial cells compared to buffer-treated (HBG) and completely untreated cells. They were seeded in 96 well plates, 10000 cells/well in triplicates. The fluorometric read-out was done with the supernatant.

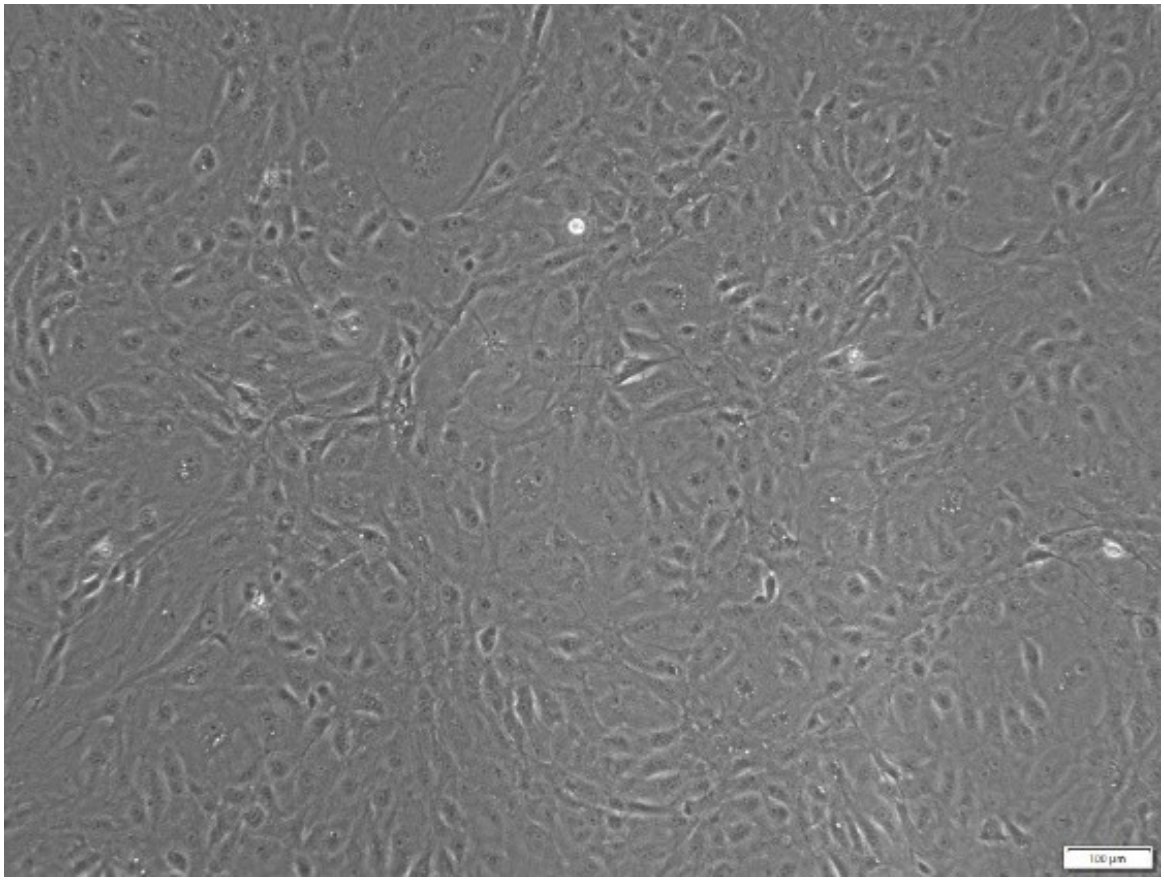

Figure 21. *Porcine endothelial cells observed in phase contrast*
P.8, picture taken on 15.01.2021. More pictures and a timeline of PEC alteration can be found in the appendix (see Figure 33 in 8.2).

BPEI showed the least influence on cell viability, in contrast to LPEI3kDa and the crosslinked PEI. C-LPEI 1.0 shows the highest toxicity of all, especially in higher concentrations, as well as C-LPEI HB. The dose-dependent effect does not apply to the formulations containing LPEI3kDa, C-LPEI 0.5 and LF3K (as shown in Figure 22), they rather show a slightly higher cytotoxicity when used in the lower concentration of 80 pmol.
As numerous studies already demonstrated, particles formed with linear PEI have quite a cytotoxic potential, which this thesis underscores. This effects are clearly dependent on the molecular weight (Godbey et al. 1999) which this experiment underlines as well, because the highest cytotoxic effect results with using C-LPEI 1.0. In contrast to cancer cells like HeLa (Kunath et al. 2003), primary cells like endothelial cells seem to be less sensible to BPEI, as the data below show. Interestingly, the buf-

fer-treated cells slightly got affected by their treatment. Presumably, the buffer treatment or even the removal of medium before adding the cell viability reagent, lead to damage or apoptosis of PEC.
Since this experiment was only performed once and the prior optimization of the resazurin assay for PEC was challenging due to difficulties to reach the desired R-square in the optimization process (see data in appendix), further investigation is recommended to get reproducible data.

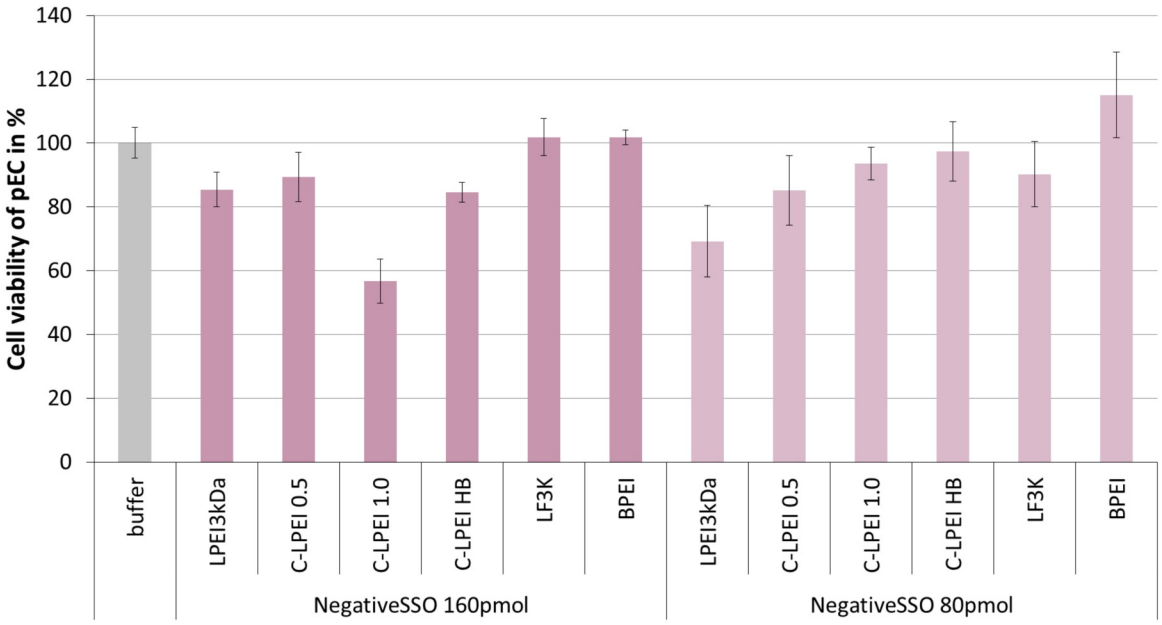

Figure 22. *Cell viability in % of NegSSO-treated porcine endothelial cells compared to buffer treated PEC.*
Cells were seeded at a density of 10000 cells/well in triplicates, followed by 24 hours of incubation and polyplex treatment with an incubation duration of 4 hours. Two different concentrations were used: dark red columns show cell viability for polyplex concentration of 160 pmol SSO/well, light red values show cell viability of polyplex concentration of 80 pmol SSO/well. The graph shows the mean ± standard deviation of n=1 experiment.

4.3. Characterization of basal firefly signal in HeLa pLuc705

HeLa pLuc075 have low or no luciferase activity due to the aberrant splicing of its pre-mRNA. Based on this, an investigation of fold increase gained by splice-correction was carried out via firefly luciferase assay. This aberrant mRNA splicing can be corrected with LucSSO, eventually resulting in the expression of luciferase protein. Before the splice-correction rate can be observed, a read-out of the basal luciferase signal in HeLa wt und pLuc705 was performed. Cells were seeded in different numbers (see Figure 10) and in triplicates in 96 well plates for performing the FFLuc/BCA assay. The determination of the luciferase signal was carried out with a Luminometer, plotting RLU against µg protein.

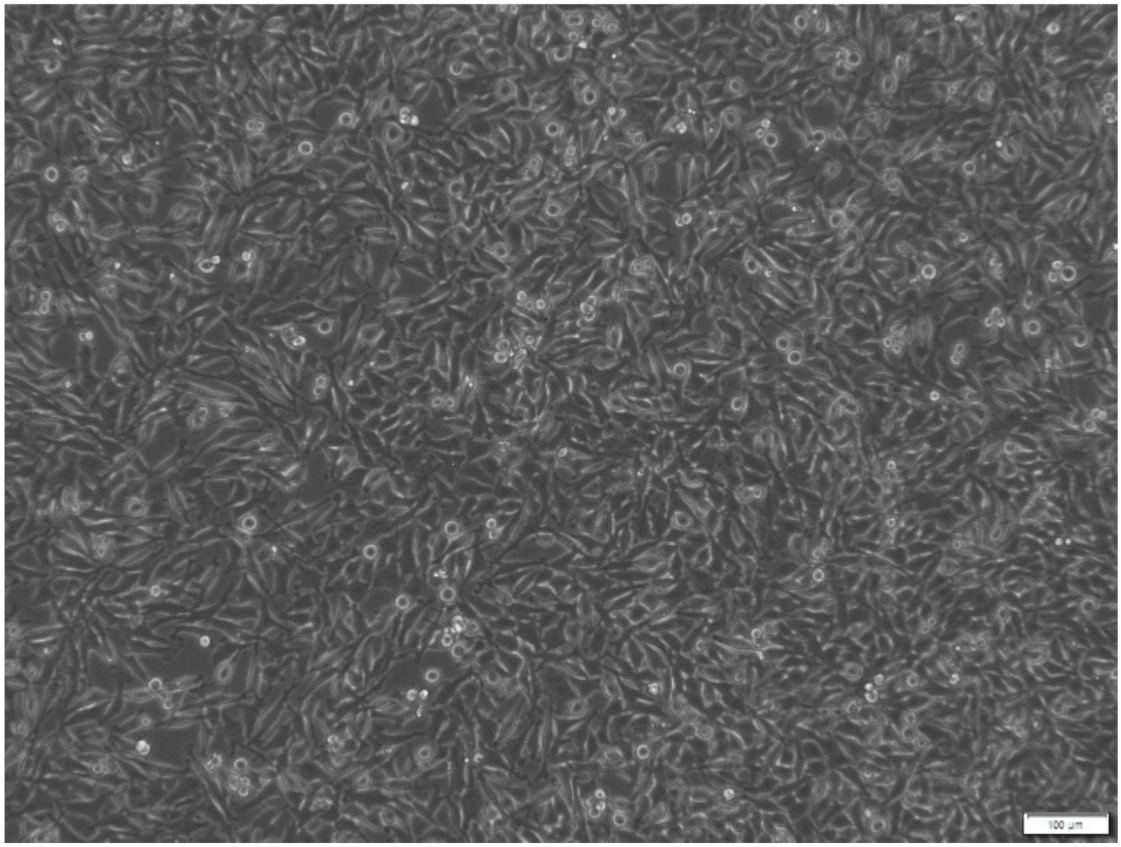

Figure 23. *HeLa pLuc705 observed in phase contrast.*

As expected, HeLa wt show no luciferase expression at all. The basal luciferase signal of HeLa pLuc705 ranges between 100-1000 RLU/1 µg protein, independently of the seeded cell number, as the normalized data show in Figure 24. Kang et al. also demonstrated, that these signals are just background levels of luciferase expression in pLuc705.

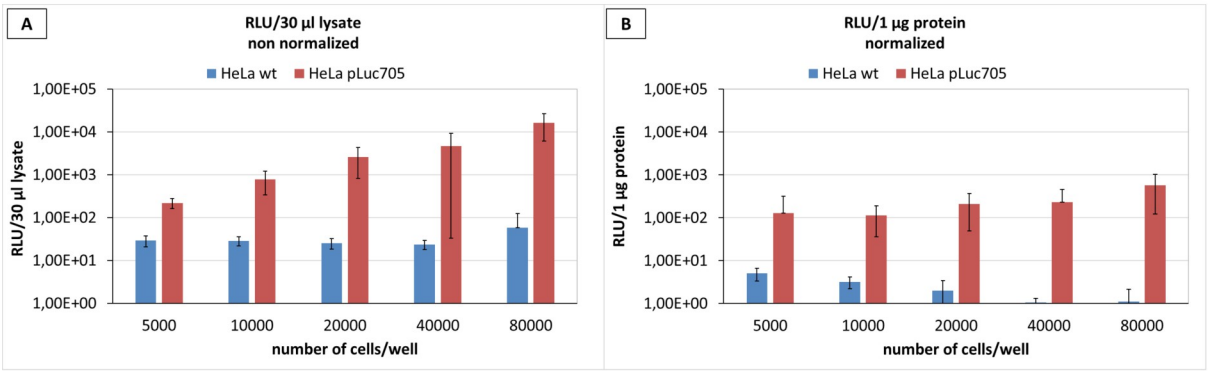

Figure 24. *Non normalized (A) and normalized (B) basal luciferase activity in HeLa pLuc705 compared to HeLa wt*

via FF-Luc/BCA-assay. HeLa wt/pLuc705 were seeded in above mentioned cell densities in triplicates, followed by cell lysis and firefly luciferase read-out. Corresponding protein amounts are to be found in the appendix in 8.5. The data show the mean ± standard deviation of n=2 experiments.

4.4. Multiplexing: testing splice correction by polyplexes and combining with cytotoxicity

4.4.1. Investigating splice correction efficiency of different polyplexes

The main aim of this thesis was to investigate splice-correction using polyplexes consisting of antisense nucleotides complexed with different types of cationic polymers, which have different qualities concerning transfection efficiency and cytotoxicity. Both properties were examined simultaneously in three experiments via firefly luciferase assay and resazurin assay.

The polymers investigated include different polyethylenimines (PEI), namely LPEI3kDa, crosslinked PEI 0.5, crosslinked PEI 1.0, crosslinked PEI HB and as positive controls Lipofectamine 3000 and branched polyethylenimine (BPEI). As a model cell line for the *in vitro* investigation of splice-correction HeLa pLuc705 were used, which are stably transfected with the reporter plasmid pLuc075, resulting in a defective human beta-globin intron 2 (IVS2-705) (Resina et al. 2007). This mutation leads to an aberrant splicing-site, followed by production of a dysfunctional protein. Antisense-oligonucleotides like luciferase splice-switching oligonucleotides (LucSSO) can inhibit the mutation and lead to correct splicing, hence the biosynthesis of a functional luciferase protein.

These LucSSO-treated HeLa pLuc705 show increased luminescence, compared to the NegSSO-treated, untreated and buffer-only treated cells (see Figure 25), which indicates a correction of the divergent splicing-site. Interestingly, the RLU values of NegSSO are higher than the basal Luciferase expression in HeLa pLuc705 (see Figure 24), presumably because of the decreased protein amount after transfection. To detect this effect on cell viability, resazurin assay was performed with the supernatant before cell lysis as described in the following sections.
To detect splice correction, the RLU values of the LucSSO polyplexes must be divided by those of the corresponding NegSSO polyplexes to obtain the fold increase in splice correction, which is shown in Figure 26.

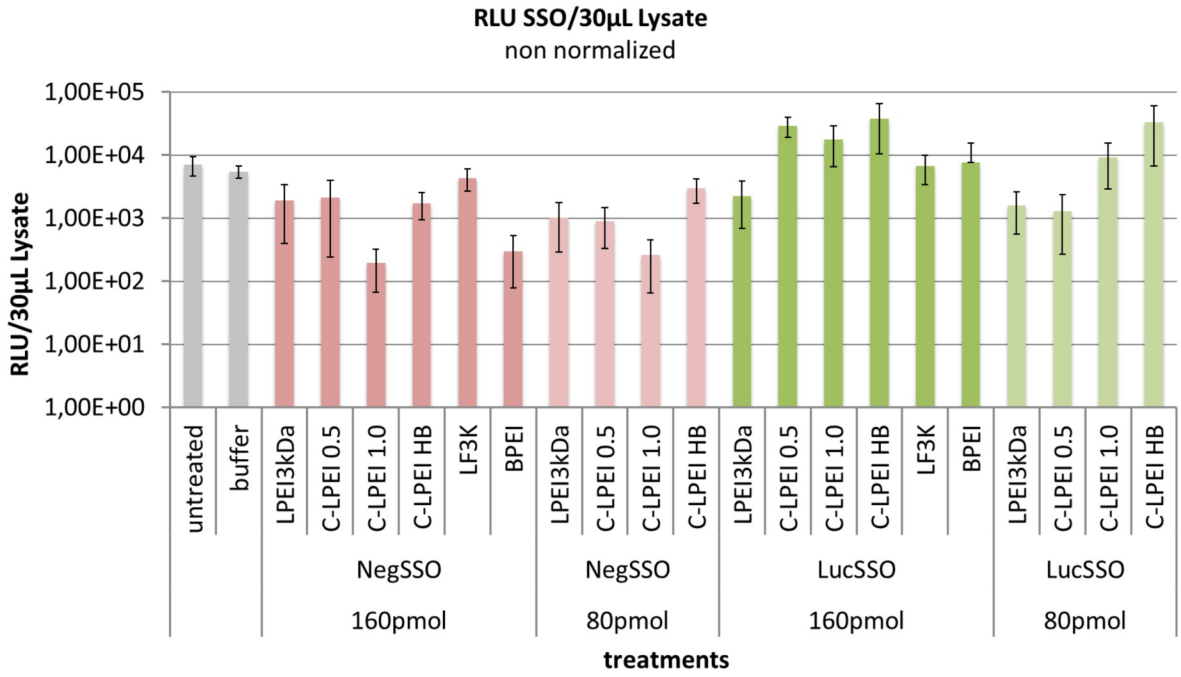

Figure 25. *RLU of polyplex-treated - based on (red) Neg and (green) LucSSO - HeLa pLuc705.*
HeLa pLuc705 were seeded at a density of 10000 cells/well in triplicates, followed by 24 hours of incubation and polyplex treatment with an incubation duration of 4 hours. Luminescence data is not normalized and shown for 30 µl lysate of Px-treated HeLa pLuc705. The first two grey columns show the RLU for completely untreated and buffer-only treated cells. Dark shaded columns show RLU of 160pmol SSO/well treated and light shaded columns RLU of 80 pmol SSO/well treated cells. The data show the mean ± standard deviation of n=3 experiments.

It is striking that the efficiency of correcting an aberrant splicing-site is highest when using crosslinked polyethylenimines (see Figure 26). C-LPEI 1.0 and C-LPEI HB show the highest fold increase independently of the used polyplex concentration, C-LPEI 0.5 works efficiently when used in 160 pmol LucSSO/well compared to 80 pmol LucSSO/well. In contrast, LPEI 3kDa has a dose-independent, but low ability to increase splice correction, as expected. Lipofectamine 3000 (LF3000) and branched polyethylenimine (BPEI) were used as positive controls and used as a reference of transfection efficiency. Treatment with Lipofectamine 3000 led to low splice-correction compared to crosslinked polymers, but BPEI shows similar fold increase like crosslinked PEI.

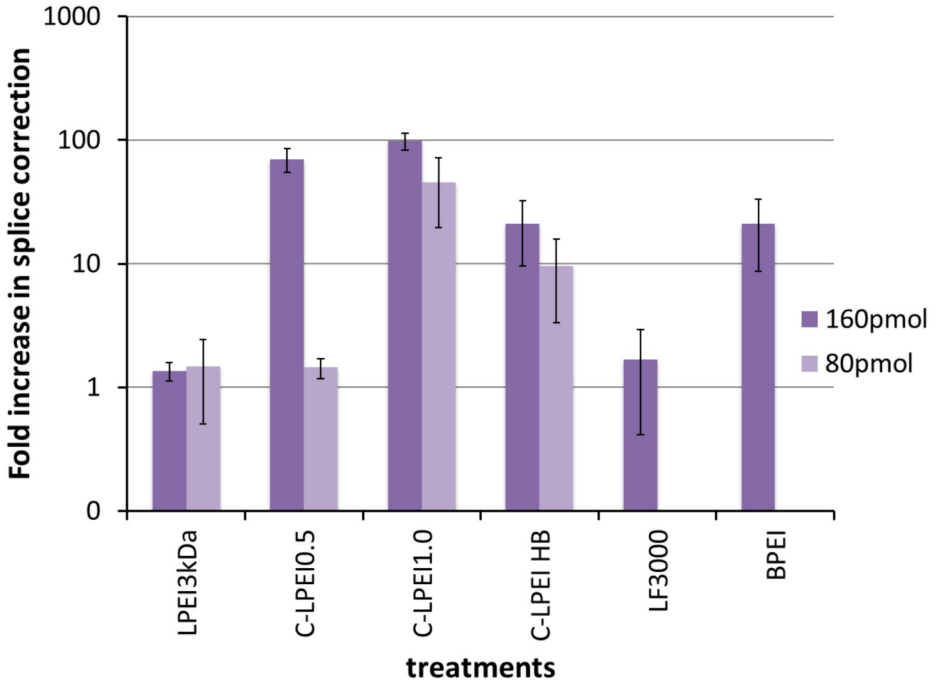

Figure 26. *Fold increase in splice correction of LucSSO-treated HeLa pLuc705.*
Dark shaded columns show the fold increase of cells treated with LucSSO concentration of 160 pmol/well, light shaded columns the fold increase of cells treated with LucSSO concentration of 80 pmol/well. The data show the mean ± standard deviation of n=3 experiments.

To determine if there was a statistically significant difference in splice correction ability between the different polymers at 160 pmol/well, a t- and Mann-Whitney-U-test was performed retrospectively.

160pmol/well	t-test		Mann-Whitney-U	
C-LPEI 0.5 vs. LPEI3kDa	n.s.	n.s.	0,05	0,01
C-LPEI 1.0 vs. LPEI3kDa	0,05	0,01	0,05	0,01
C-LPEI HB vs. LPEI3kDa	0,05	0,01	0,05	0,01
C-LPEI 0.5 vs. C-LPEI HB	n.s.	n.s.	n.s.	n.s.

The table shows the different statistical tests used and their significance levels.

As shown in Figure 27, the t-test results show that C-LPEI 1.0 and C-LPEI HB lead to higher transfection efficiency than LPEI3kDa. The difference between C-LPEI 0.5 and LPEI3kDa is not significant, as well as C-LPEI 0.5 and C-LPEI HB. The U-test (see Figure 28) shows slightly different results than the t-test.

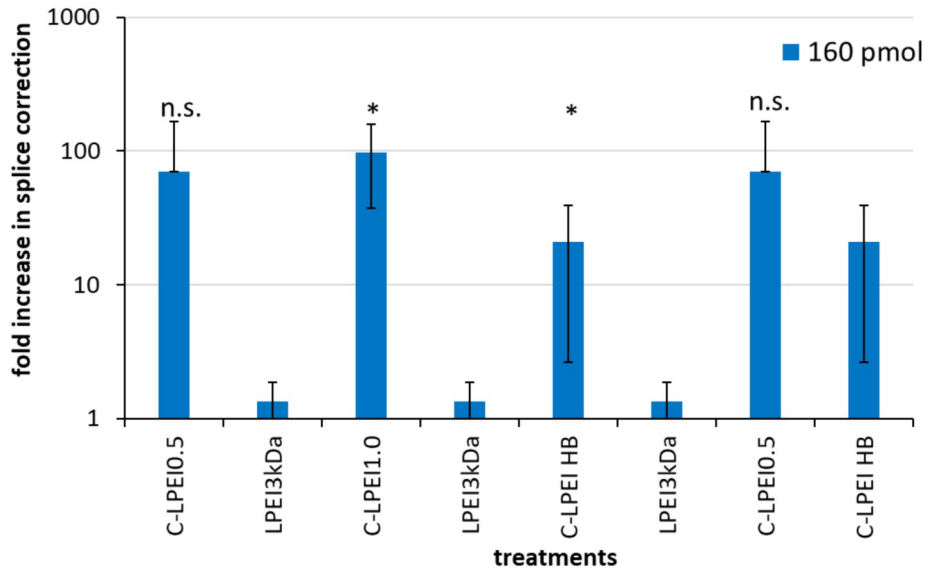

Figure 27. *t-test of splice-correction by using different cationic polymers.*
*P<0.05 and P<0.01, n.s. not significant at both. Values represent the mean ± standard deviation of n=3 experiments.

Also, here it shows that using C-LPEI 0.5 is not superior to using C-LPEI HB. But compared to the usage of LPEI3kDa, all crosslinked polymers are significantly superior to enable efficient splice-correction.

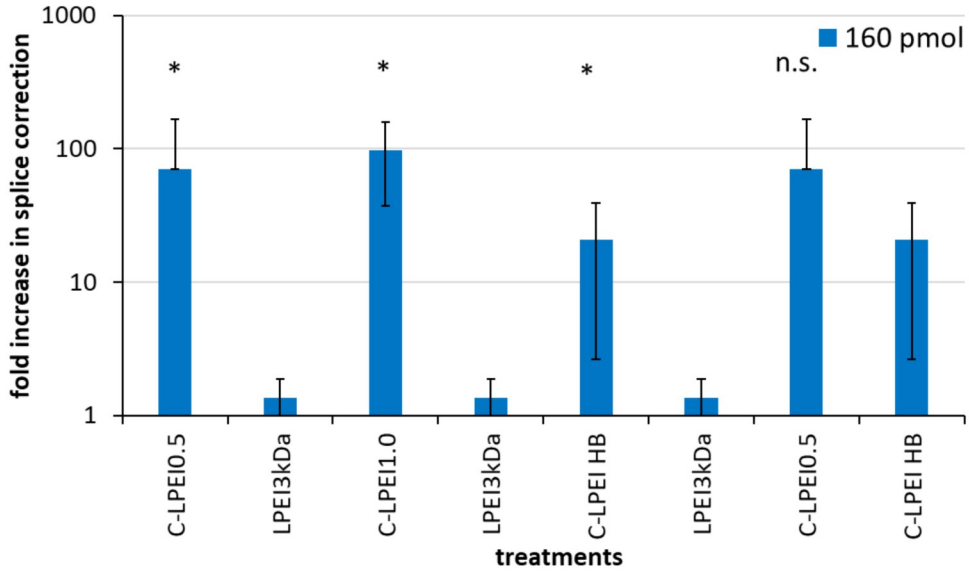

Figure 28. *Mann-Whitney-U-test of splice-correction by using different cationic polymers.*
*P<0.05 and P<0.01, n.s. not significant at both. Values represent the mean ± standard deviation of n=3 experiments

Overall, the results of the comparison of different cationic polymers regarding splice correction rate were as expected and demonstrated in various publications. Previous studies have shown that linear polyethylenimine (LPEI3kDa) has a much lower transfection efficiency compared to its crosslinked de-

rivatives (C-LPEI 0.5, 1.0 and HB) and branched polyethylenimine (Bonner et al. 2013; Breunig et al. 2008; Breunig et al. 2007). Sundaram et al. proved in their study, that the molecular weight of polymers is substantial for successful ASO delivery, which possibly explains the lack in transfection efficiency for LPEI3kDa compared to the other polymers examined. Furthermore, the positive controls, the commercial kit Lipofectamine 3000 and non-crosslinked BPEI differ from each other. Whereas BPEI is well known for its successful transfection ability, the commercial product does not achieve high splice correction rates compared to branched polyethylenimines and crosslinked polyethylenimines. As previously stated, BPEI is a recognised polymer in polyplexing, however, its high cytotoxicity has resulted in further investigation into crosslinked polyethylenimine. These derivatives can display comparable transfection efficiency while maintaining lower levels of toxicity (Bonner et al. 2013). The crosslinked polyethylenimines partly differed in their behaviour *in vitro*, which probably is due to the difference in the level of crosslinking. C-LPEI 1.0 which has the highest crosslinking degree, achieves splice-correction like the “less” crosslinked C-LPEI 0.5. In contrast, C-LPEI HB which has a similar crosslinking as C-LPEI 0.5 shows less efficient splice correction, but a dose-independent effect compared to its pendant and C-LPEI 1.0. The degree of crosslinking appears to not influence transfection efficiency much, because the difference between C-LPEI 0.5/HB and C-LPEI 1.0 is marginal. Although crosslinking PEIs results in better stability of the complex and transfection, compared to linear PEIs, it is conceivable that C-LPEI 1.0 with higher degree of crosslinking is degraded earlier in the cytoplasm as intended, since crosslinked PEIs are known for their rapid *in situ* degradation (Das et al. 2015).

4.4.1. Resazurin assay for determining cell viability

The cell viability of HeLa pLuc705 treated with splice-switching oligonucleotides in different concentrations was examined in three independent experiments (Figure 29). In general, these experiments demonstrated that cytotoxicity seems higher in NegSSO compared to LucSSO, except for dosages of 80 pmol C-LPEI HB where it is slightly increased compared to the dosage of 160 pmol. For C-LPEI 1.0 and C-LPEI HB a dose dependent cytotoxicity could be observed, since the RFU values are higher when using a dosage of 80 pmol/well. LPEI3kDa and C-LPEI 0.5 showed contradictory results, with cytotoxicity being more pronounced at the lower dose of 80 pmol, particularly with C-LPEI 0.5. It is striking that the positive control, Lipofectamine 3000, has the least influence on cell viability compared to buffer-only treated cells, whereas branched polyethylenimine shows enormous decreased viability of HeLa pLuc705. Also crosslinked polyethylenimine 1.0 has an outstanding negative effect on cell viability which seems dependent on the used concentration. The other crosslinked polyethylenimine 0.5 and HB show a cytotoxic effect, but it is – independently from the concentration – less pronounced than in C-LPEI 1.0. Linear polyethylenimine 3kDa has cytotoxic properties as well, but the effect seems higher when the lower concentration of 80 pmol/well was used.

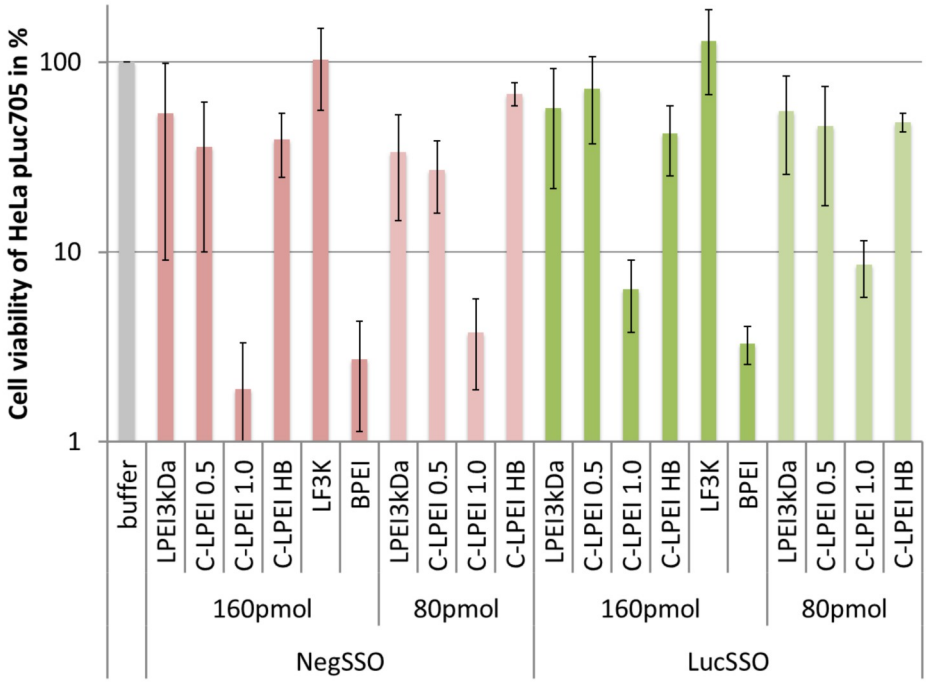

Figure 29. *Cell viability in % of polyplex-treated - based on (red) Neg and (green) LucSSO - HeLa pLuc705.* The grey column shows cell viability of buffer treated cells, which was taken as a reference. Dark shaded columns show cell viability of 160 pmol SSO/well treated and light shaded columns the cell viability of 80 pmol SSO/well treated HeLa pLuc705 compared to buffer treated cells. The data show the mean ± standard deviation of n=3 experiments.

Retrospectively, a t-test was performed to examine if the postulated difference in cytotoxicity of C-LPEI 0.5 and C-LPEI HB is significant. The reason for comparing these two components is that they have the same molecular weight, and additionally both showed the most promising results concerning splice correction and cytotoxicity. The test resulted, that there is no significant difference on both significance levels P<0.05 and P<0.01 (see Figure 30).

	160pmol/well	t-test	
	Sign. Level	0,05	0,01
LucSSO	C-LPEI 0.5 vs. C-LPEI HB	n.s.	n.s.
NegSSO	C-LPEI 0.5 vs. C-LPEI HB	n.s.	n.s.

Table shows the t-test done with the corresponding significance levels.

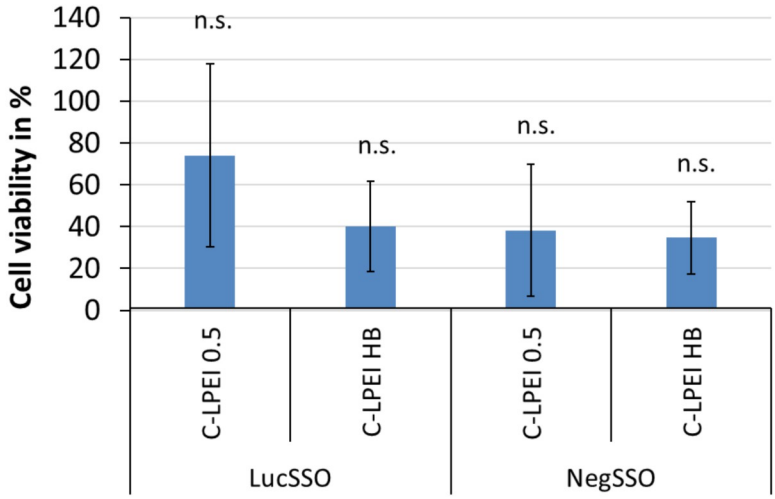

Figure 30. *t-test: Cell viability of Px treated HeLa pLuc705.*
n.s. not significant at both. The data show the mean ± standard deviation of n=3 experiments.

As Kunath et al. already demonstrated, BPEI shows the strongest cytotoxic effect, but at the same time a high transfection efficiency *in vitro*, which creates the need for investigating the properties of crosslinked PEIs to erase the disadvantage of cytotoxicity. Also, the treatment using C-LPEI 1.0 shows enormously decreased cell viability, compared to other crosslinked polymers used in this study. These results contradict the findings of the study by Bonner et al., where it could be proven, that the increased cell viability when using crosslinked PEI is a result of high cross-linking ratio and hence charge neutralization. Toxicity of cationic polymers like PEI is caused by the high rate of positively charged amino groups on the backbone of the polymer, which can lead to interactions between nanoparticles and cell membranes (McConnell et al. 2016). One possible explanation for the decreased cell viability of HeLa pLuc705 may be the edge-effect. It could be demonstrated, that in 96 well plates the wells on the outer edge tend to have more evaporation of e.g., medium, than the central ones. It is possible, that because of evaporation, HeLa pLuc705 reduced their metabolic activity, because the volume of culture-medium decreased (Mansoury et al. 2021; Rampersad 2012). It is reasonable to assume that C-LPEI 1.0 exhibits higher toxicity than its less branched derivatives. It is known that the release of the payload when using crosslinked cationic polymers is increased, which is associated to increased intracellular stress. Additionally, long incubation (24 hours) of polyplexes using cationic polymers as a complexing reagent leads to apoptosis via intrinsic pathway (Beyerle et al. 2010).
The other crosslinked PEI 0.5 and HB act moderately cytotoxic while remaining efficient splice correction as expected, whereas LPEI3kDa shows a moderate effect on cell viability too, but in contrast has poor transfection efficiency. The lipid-based commercial agent Lipofectamine 3000 showed no cytotoxic effect at all, but also has a poor splice-correction ability compared to the cationic polymer-based polyplexes.

5. Conclusion

Antisense therapy gains more and more importance nowadays since selected antisense oligonucleotides got approved by FDA and *in vitro* studies in the past showed promising results. Additionally, several antisense drugs for various diseases are in the pipeline, as detailed in reported studies (Moumné et al).

This thesis successfully demonstrated the possibility of simultaneously detecting *in vitro* splice-correction of the aberrant luciferase splicing site by PEI-SSO polyplexes and their associated cytotoxicity in HeLa pLuc705 cells.

Determining cell viability with resazurin assay is a common and reliable method which was optimized in this work. The major advantage of preparing a housemade resazurin solution is the costs, because resazurin salts are inexpensively to purchase. Compared to commercial products the linearity is similar in HeLa pLuc075 and PEC. It also could be shown that the signal intensity is clearly decreased in both cell types. The fact that it must be prepared freshly for every experiment due to its low stability creates extra efforts which are time-consuming and had led to the decision to use Alamar Blue HS for the further experiments which have been done.

In conclusion, it is relatively clear that crosslinked polyethylenimines are superior in terms of splice-correction ability compared to non-crosslinked controls branched polyethylenimine and lipid-based Lipofectamine, as well as linear polyethylenimine. These findings underline the potential that the usage of cationic polymers have in antisense oligonucleotide-based therapy, or gene therapy in general, and are a promising tool in future biomedical research to either study further *in vitro* effects or even perform *in vivo* experiments in preclinical studies. Nevertheless, it is recommended that further investigations concerning different polyethylenimines, especially crosslinked derivates, are performed, because this thesis only gave a brief insight into the topic.

The cell viability experiments resulted in partly contradictory results, compared to findings of Breunig et al. While branched polyethylenimine showed cytotoxicity as expected, the crosslinked PEI 1.0 has surprisingly high cytotoxicity, even more than BPEI. As already mentioned in the results and discussion section, there are numerous assumptions on the reasons for decreased cell viability. For future experiments, it is recommended to alter the experimental design. For example, *in vitro* investigation of different cationic polymers could be performed with different cell numbers per well, to detect differences in cytotoxicity associated with cell density. It is also interesting to compare only crosslinked polyethylenimines to gain knowledge about the influence of the crosslinking level on cell viability. Additional methods to examine cell viability may include microscopical investigation of the viability of cells or even flow cytometry.

6. List of figures

7. References

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8. Appendix

8.1. Investigation of Firefly and Gaussia expression in Geneticin-enriched HEK3PLuc

In the beginning of this thesis, one goal was to investigate the activity of Hedgehog and Notch signalling pathway in Geneticin-enriched HEK293T3PLuc compared to HEK293Twt via Firefly luciferase/BCA- and Gaussia luciferase assay. The initial work that was done, has been continued by Julia Moldaschl.

8.1.1. Normalized data of Firefly luciferase expression in HEK3PLuc

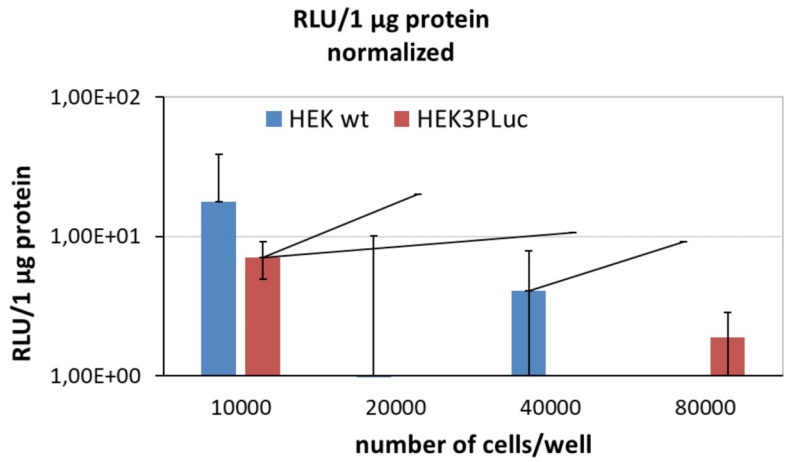

Figure 31. *Normalized Hedgehog pathway activity estimation in HEK293T 3P-Luc cells* (as compared to HEK293T wt cells) via Firefly luciferase read-out. HeLa pLuc705 were seeded at above mentioned cell densities and followed by lysis and using the method with scratching. Normalized Firefly luciferase expression data after 24h. The data shows the mean ± standard deviation of n=2 experiments.

8.1.2.Data of Gaussia luciferase expression in HEK293T wt and 3PLuc

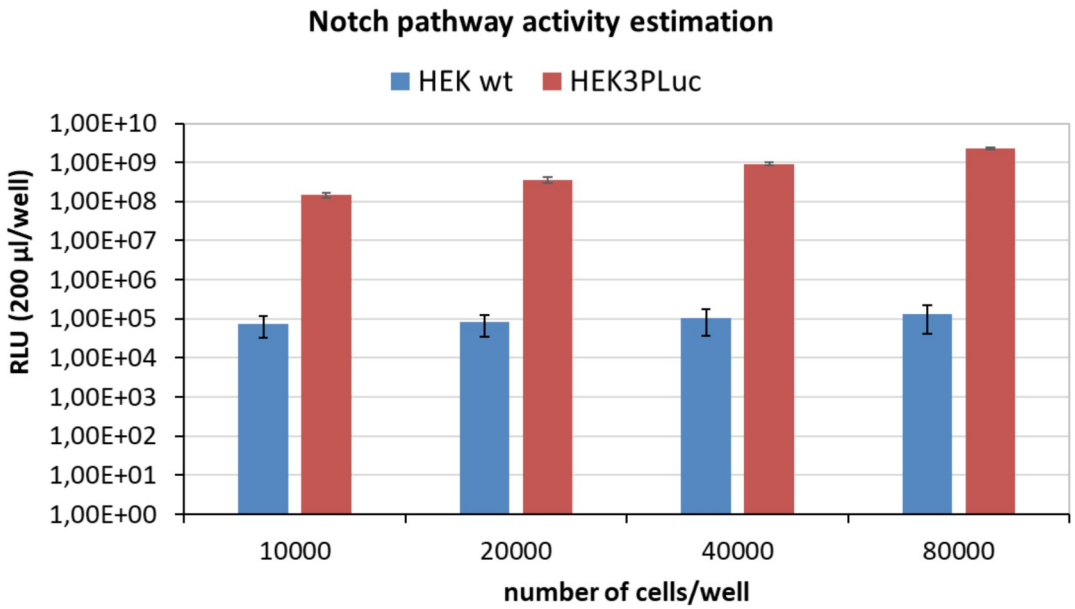

Figure 32. *Notch pathway activity estimation in (blue) HEK293Twt and (red) 3P-Luc cells* via Gaussia luciferase read-out. HEK293T were seeded at above mentioned cell densities in triplicates. Non-normalized Gaussia luciferase expression data after 24h. The data show the mean ± standard deviation of n=2 experiments.

8.2. Morphological observations in porcine endothelial cells

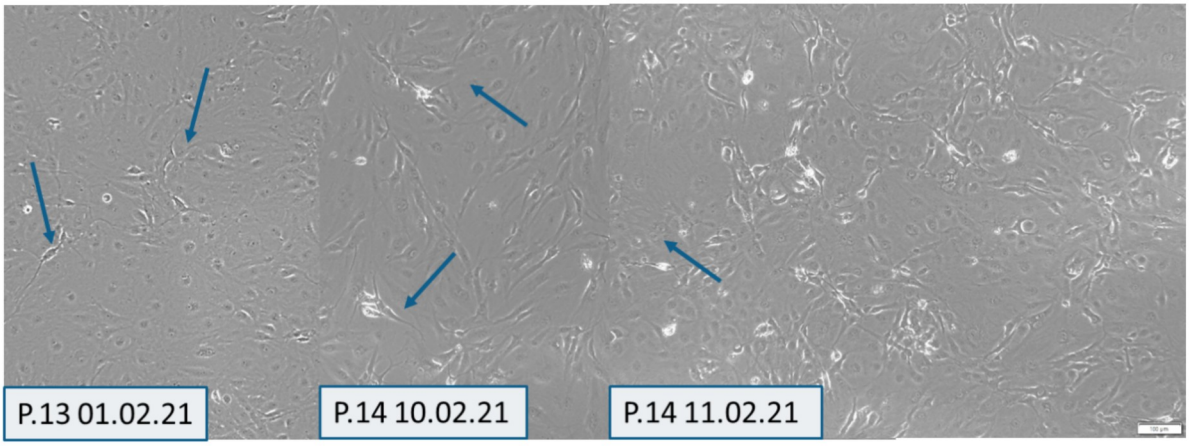

Figure 33. *Timeline of morphological changes of porcine endothelial cells visible under phase contrast.* Alteration of cells from cobblestone appearance to fibroblast-like cell growth over time. From left to right: P.13 01.02.2021, P.14 10.02.2021, P. 14 11.02.2021

8.3. Optimization of cell viability assays

8.3.1. Optimization of MTT assay

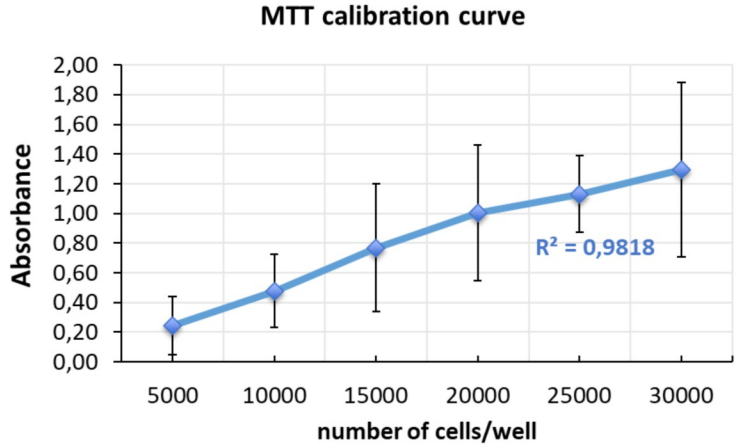

Figure 34. *MTT Calibration curve with Absorbance plotted against number of HeLa pLuc705 seeded.*
HeLa pLuc705 were seeded in above mentioned cell densities in triplicates, followed by 24 hours of incubation and MTT assay. The data show the mean ± standard deviation of n=3 experiments.

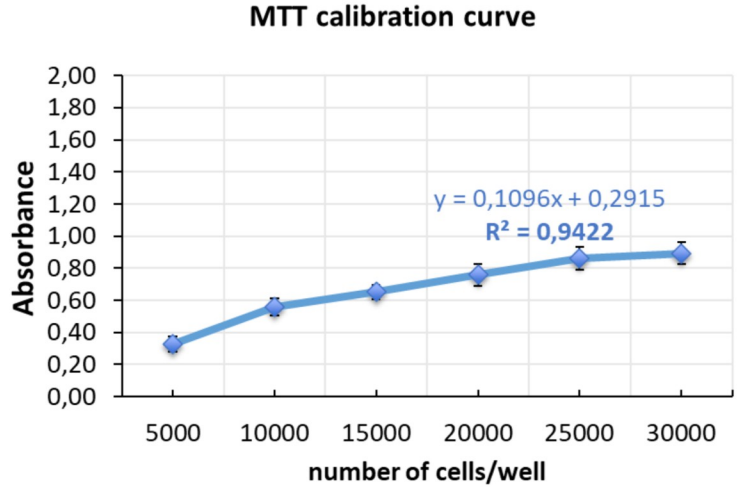

Figure 35. *MTT Calibration curve with Absorbance plotted against number of PEC seeded.*
Cells were seeded in above mentioned cell densities in triplicates, followed by 24 hours of incubation and MTT assay. The data show the mean ± standard deviation of n=2 experiments.

8.3.2. Optimization of Resazurin assay

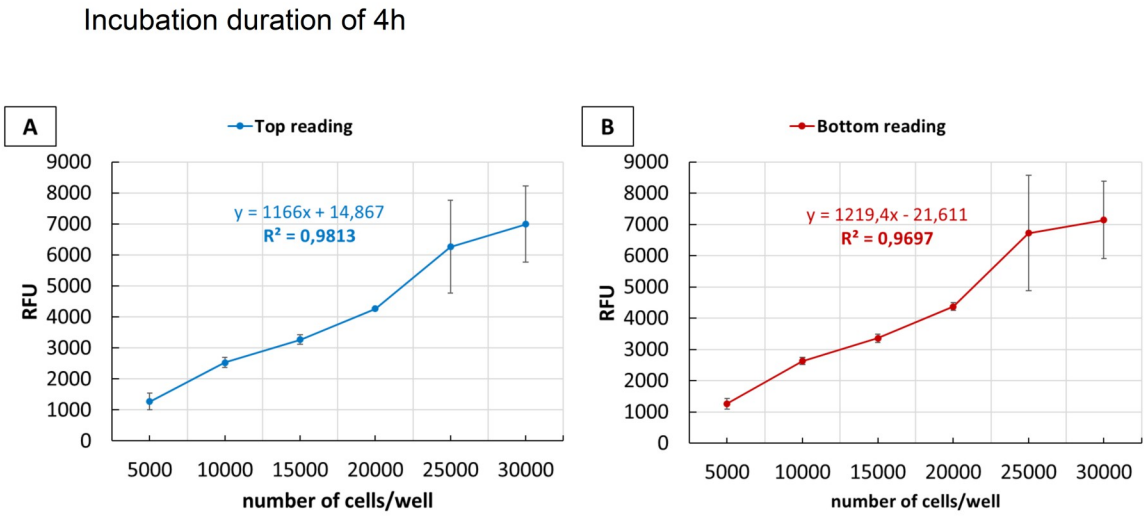

Figure 36. *Incubation duration 4h: Resazurin calibration curves with relative fluorescence units plotted against number of HeLapLuc705 seeded (per well).*
Testing incubation with housemade Resazurin solution with an incubation duration of 4h in (A) top and (B) bottom reading mode. HeLa pLuc705 were seeded in above mentioned cell densities in triplicates, followed by 24 hours of incubation and resazurin treatment with an incubation duration of 4 hours. The data show the mean ± standard deviation of n=2 experiments.

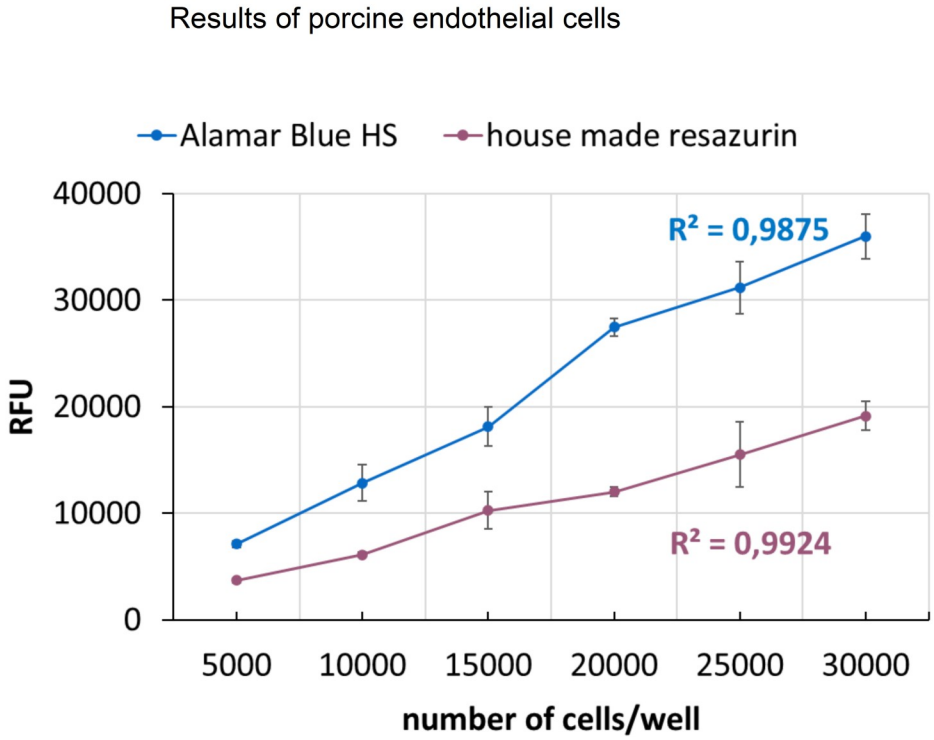

Figure 37. *Comparison of Alamar Blue HS and housemade resazurin solution performed with PEC.*
Correlation between measured fluorescence and number of cells seeded. Each graph shows triplicates of one experiment.

8.4. Cytotoxicity of polyplexes in porcine endothelial cells

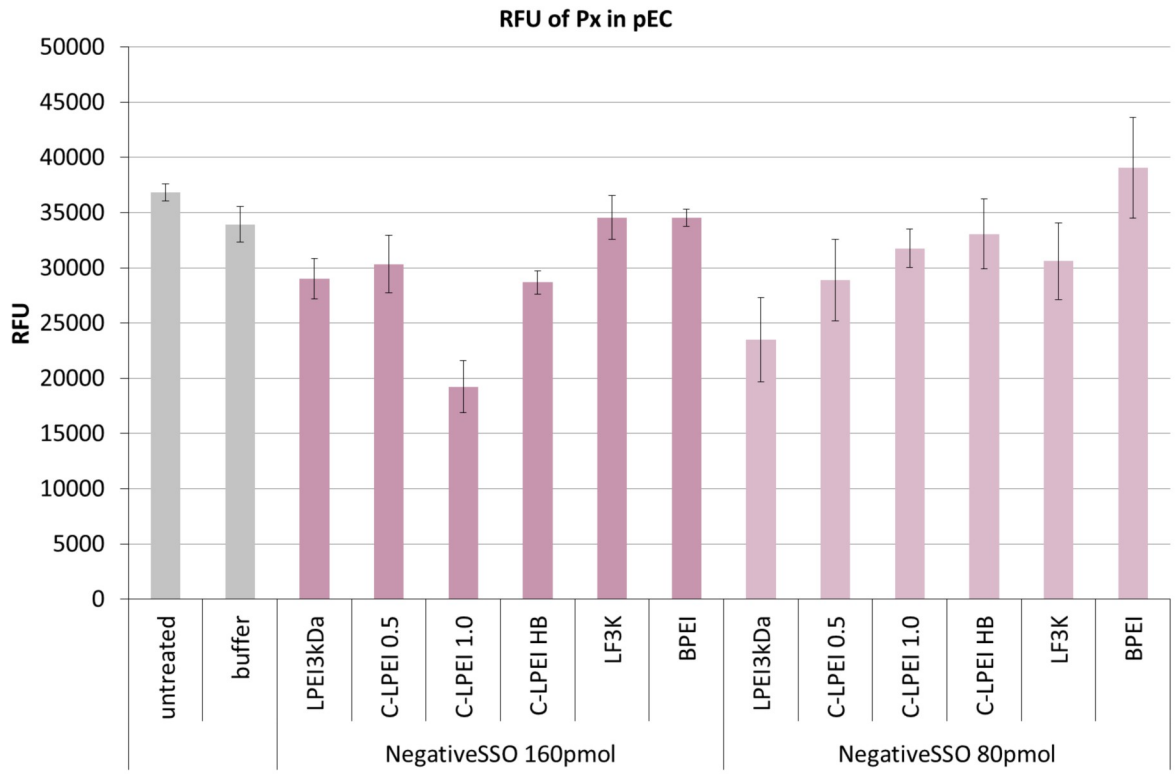

Figure 38. *Relative fluorescence units of NegSSO-treated porcine endothelial cells* compared to buffer treated PEC. Two different concentrations were used: dark shaded columns show RFU for polyplex concentration (A) of 160 pmol SSO/well, light red values show RFU of polyplex concentration (B) of 80 pmol SSO/well. The data show the mean ± standard deviation of n=1 experiment.

8.5. Characterization of basal Firefly luciferase signal in HeLa pLuc705

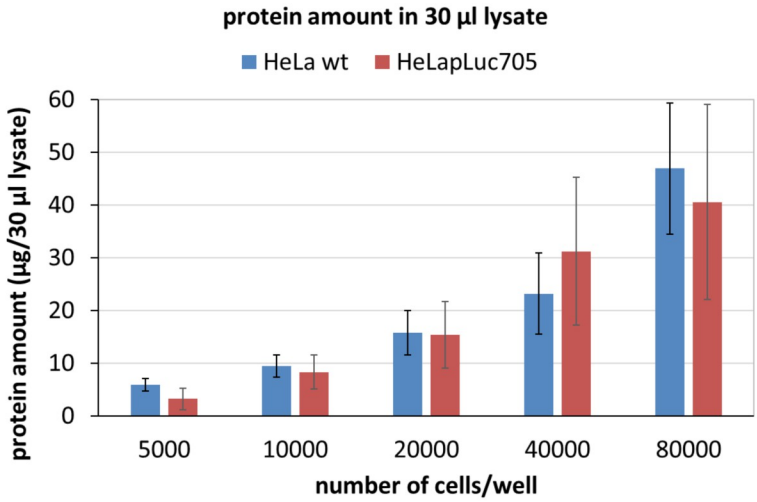

Figure 39. *Corresponding protein amounts of Firefly luciferase in HeLa pLuc705* compared to HeLa wt. Cells were seeded in above shown cellnumbers/well in triplicates. The graph shows the mean ± standard deviation of n=2 experiments.

8.6. Cytotoxicity of polyplexes in HeLa pLuc705

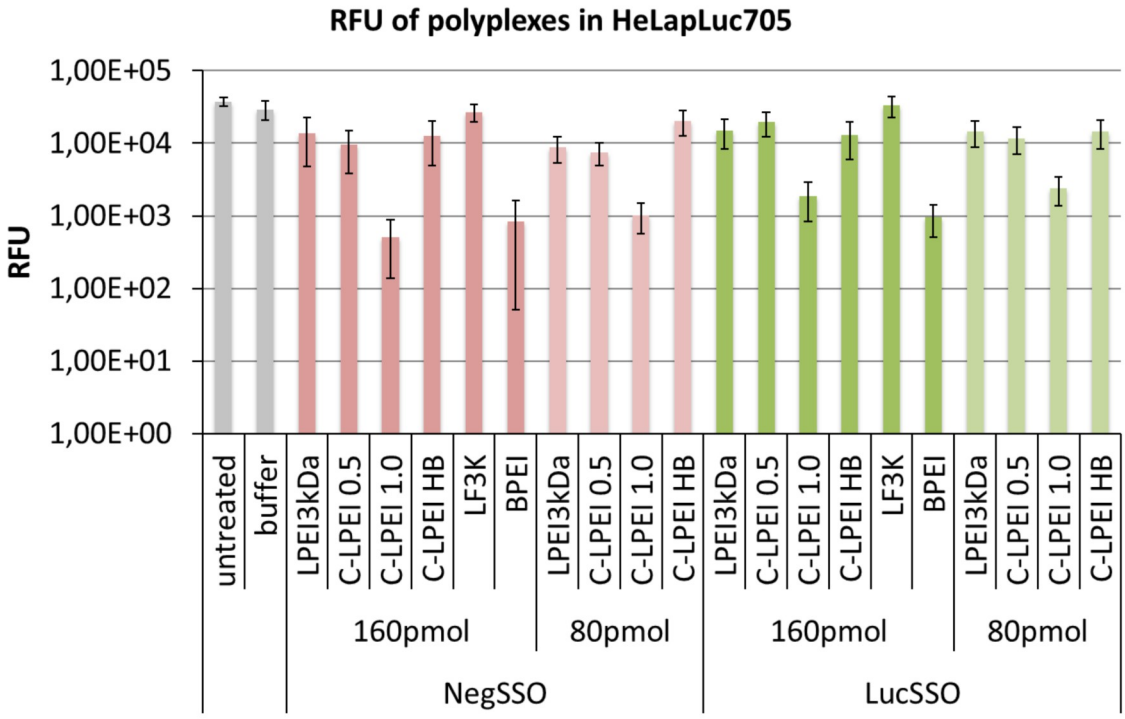

Figure 40. *RFU of polyplex treated HeLa pLuc705 per well.*

Fluorescence data is not normalized and plotted as average from three independent experiments. The first two grey columns show the RFU for completely untreated and buffer-only treated cells. Dark shaded columns show RLU of 160pmol SSO/well treated and light shaded columns RLU of 80 pmol SSO/well treated HeLa.

8.7. Testing splice correction via PCR

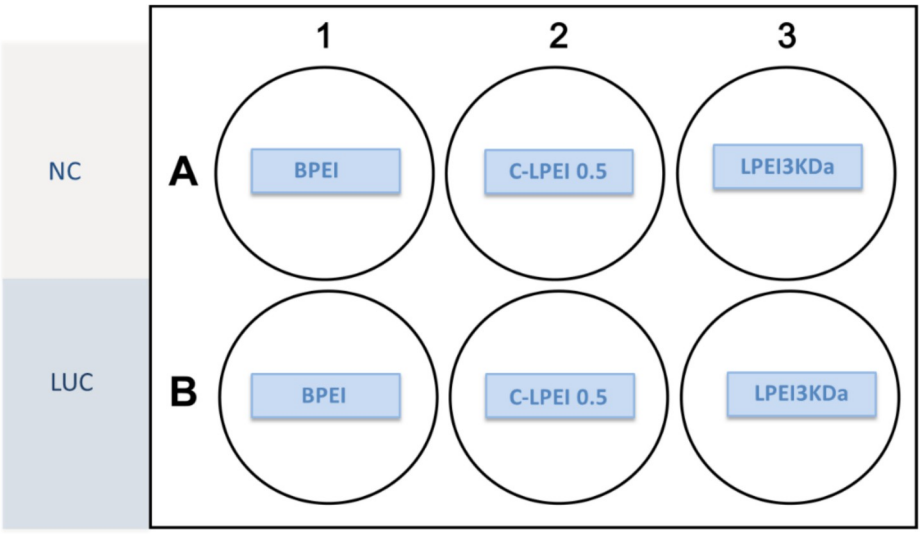

Figure 41. *Schematic overview of a 6 well plate used for PCR analysis.*

Five hundred thousand HeLa pLuc075 were seeded in each well. Cells were transfected with 640 pmol/well negative SSO (A) and Luc SSO (B) polyplexes (N/P ratio of 9) using either BPEI, C-LPEI 0.5 or LPEI3kDa as polymers. After cell harvesting, the rate of splice-correction got analysed via PCR by Arian Tahmaseb-Zamanian.