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„Doch Forschung strebt und ringt, ermüdend nie,

Nach dem Gesetz, dem Grund, Warum und Wie.“

(Johann Wolfgang von Goethe)

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1. Abstract

A lot of alternatives to conventional therapies for cancer have been researched in recent years because of many undesired side effects or not enough specificity of used agents. Especially in the field of gene therapy successful improvements have been made.

Generally, gene delivery vectors can be divided into two groups, namely viral and non – viral vectors. In this work, we focused on non – viral gene delivery based on linear Polyethylenimine (LPEI) and LPEI-PEG-Peptide conjugates for Epidermal Growth Factor Receptor (EGFR) – mediated transfections. LPEI – plasmid DNA (pDNA) complexes, so-called polyplexes, can be used to deliver genetic material (e.g. pDNA) into cells.

In particular, we focused on testing of the peptide-PEG-LPEI conjugates with GE11 – and CLARLLT – peptide. An important feature of these two peptide – conjugates is that they bind to EGFR without receptor activation.
Biological evaluation of these gene delivery systems was performed on human (A549.wt and LS174T.wt) and murine (CT26.wt) EGFR – overexpressing cancer cell lines. Quality check of generated polyplexes was done by nanoparticle tracking analysis (NTA).

In the first part of the work, toxicity (various ng/well) and transfection efficiency (N/P ratio 6 or 9) of LPEI with Mw (weight average molecular weight) of 10,000 Da was tested and optimization of transfection assay was done.

In the second part EGFR targeting assays were performed in 96 – well plate format, focusing on CLARLLT as targeting component of LPEI based polyplexes. It could be shows that LPEI – PEG – CLARLLT polyplexes had a higher transfection efficiency than LPEI – PEG – GE11 in A549.wt and CT26.wt. This demonstrated that CLARLLT peptide can also be used for targeting the murine EGFR.

In the last part of the work, a cell fixation protocol was established and the cellular uptake was successfully visualized via Confocal Microscopy on A549.wt cells.

2. Zusammenfassung

Aufgrund von zahlreichen unerwünschten Nebenwirkungen oder zu geringer Selektivität der Wirkstoffe bei konventionellen Krebstherapiebehandlungen, wurde in den letzten Jahren nach Alternativen geforscht. Vor allem im Bereich der Gentherapie konnten erfolgreiche Fortschritte gemacht werden.

Im Allgemeinen können Vektoren für den Gentransfer in zwei Gruppen, virale und die nicht – virale, eingeteilt werden. Im Zuge der Diplomarbeit fokussierten wir uns auf nicht – virale Vektoren basierend auf linearem Polyethylenimin (LPEI) und LPEI – PEG – Peptidkonjugaten für EGFR – mediierte Transfektionen (EGFR: Epidermaler Wachstumsfaktor Rezeptor). Mittels LPEI – Plasmid DNA (pDNA) Komplexen, sogenannte Polyplexe, kann genetisches Material (z.B. pDNA) zielgerichtet in Zellen gebracht werden.

Ein besonderer Fokus der Arbeit lag auf der Austestung von Peptid Konjugaten mit den Peptiden GE11 und CLARLLT. Eine wichtige Eigenschaft dieser zwei Peptid- Konjugate ist es, dass sie an den EGFR binden ohne diesen zu aktivieren. Die biologische Evaluierung dieser Transfersysteme erfolgte an zwei humanen (A549.wt and LS174T.wt) und einer murinen (CT26.wt) EGFR – überexprimierenden Krebszelllinien. Die Qualität der generierten Partikel wurde mittels nanoparticle tracking analysis (NTA) ausgetestet.

Im ersten Teil meiner Arbeit wurden Toxizität (verschiedene ng/well) und Transfektionseffizienz (N/P ratio 6 oder 9) von Polyplexen mit LPEI (Molekulargewicht 10kDa getestet) und eine Optimierung des Transfektionsassays durchgeführt.

Im zweiten Teil meiner Diplomarbeit wurden EGFR – Targetingassays in 96 – Well Platten durchgeführt, wobei der Fokus auf CLARLLT als Targetingkomponente von LPEI –basierten Polyplexen lag. Mit unseren Untersuchungen konnten wir zeigen, dass LPEI – PEG – CLARLLT eine höhere Transfektionseffizienz als LPEI – PEG – GE11 in A549.wt und CT26.wt Zellen besitzt.

Weiters, haben wir bewiesen, dass CLARLLT auch für das EGFR-Targeting in murinen Krebszelllinien geeignet ist.

Im letzten Teil meiner Arbeit wurde ein Protokoll für die Zell – Fixation erstellt und die zelluläre Aufnahme von Polyplexen konnte erfolgreich an A549.wt Krebszellen mittels Konfokalmikroskop gezeigt werden.

3. Introduction

Cancer

Cancer, a general term used for a group of malignant tumor diseases (neoplasms or tumors), is still one of the leading diseases with a high mortality rate (Kang et al. 2015; Siegel et al.2013). In 2012, worldwide about 14.1 million patients were diagnosed with cancer and 8.2 million deaths caused by cancer occurred (based on GLOBOCAN estimates).

Lung cancer is the leading cancer disease occurring in adults in more developed countries (Torre et al. 2015). In less developed countries breast cancer remains with the highest mortality rate among females (Torre et al. 2015). Other frequently occurring types of cancer include prostate, colorectal, stomach, cervical and liver cancer (Torre et al. 2015). The most common cancer occurring during childhood is leukemia (Belson et al. 2007).

3.1.Tumor characteristics and its development

In normal cells, the so-called telomere gene limits the lifetime of the cell line. But cancer cells are often characterized by high expression of telomerase (Sandbox Networks 2000a-2017). This causes uncontrollable cell growth, they expand and can penetrate into healthy tissue and cause its destruction (Cooper et al. 2000).

An additional reason for cell death of healthy cells is nutritional deprivation. Normal tissue and cancer tissue are competing for nutrients. Further, cancer cells have an atypical cell structure, are often undifferentiated or unspecialized, lack contact inhibition and some can also metastasize. Tumor cells are able to grow in several layers deep into surrounding tissues and can spread via lymph or blood system to other tissues and organs (***Figure 1***).

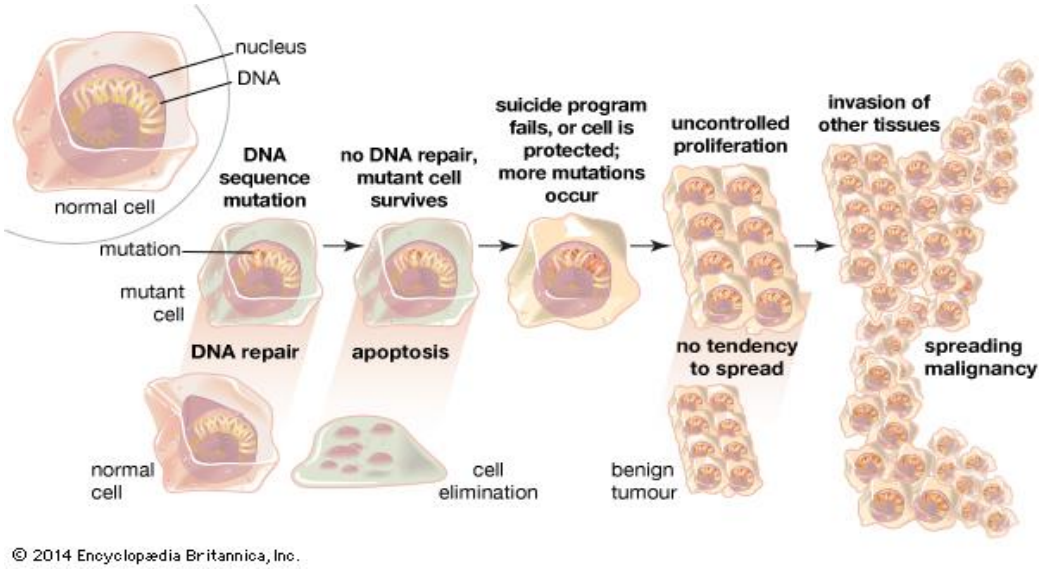

Figure 1: *Scheme of cancer development (figure from Encyclopedia Britannica, 2014). DNA repair mechanisms are essential for maintenance of DNA integrity. If the DNA repair fails, or a cell does not undergo apoptosis, severe mutations can occur. This leads to cell proliferation. Benign tumor result if the proliferation is slow and localized to the area of the origin of the tumor growth (no tendency to spread). Uncontrolled and fast cell growth, spreading and the invasion of other tissues result in malignant tumor (tendency to metastasize)*

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Repeated damage of genomic DNA (deoxyribonucleic acid) can lead to altered gene expression (e.g. of oncogenes) or mutations (e.g. in tumor-suppressor genes) (Sandbox Networks 2000c-2017). Oncogenes (such as c-ras, c-myc and v-myc) are responsible for the production of growth factors. Normally tumor-suppressor genes (such as p53, BRCA1- genes and p16) produce a negative growth factor, which signals a cell when to stop dividing (Sandbox Networks 2000c-2017). In tumor cells regulation of cell-dividing and -differentiation is disturbed, oncogenes are abnormally activated, and tumor-suppressor genes are abnormally inactivated (Sandbox Networks 2000c-2017).

However, it is assumed that several steps are necessary for the formation of tumor tissue. For the formation of an actively transformed oncogene further changes need to be added (e.g. induction by tumor promoters) (Sandbox Networks 2000c-2017).

3.2.Cancer therapy - possibilities and limitations

A goal in cancer therapy is to destroy tumor cells without harming healthy tissue (Miller et al. 2013). In recent decades, great success has been achieved in tumor therapy, like improving quality of life and prolonging lifetime of cancer patients (Ginn et al. 2013). Currently, the gold standard in cancer treatment is a combination of surgery with subsequent administration of chemotherapeutic drugs and/ or radiation therapy (Rampling et al. 2004).

In recent years, immunotherapy and targeted therapies against cancer have become increasingly important (Sandbox Networks 2000b-2017) because conventional chemotherapeutics are still associated with many problems such as development of drug resistance caused by abnormal gene expression after repeated drug administration (Kang et al. 2015; Borst et al. 2000; Gottesman et al.2002).

<u>Examples for such drug resistances developed by cancer cells are:</u>

- Efflux pumps, which can lead to reduced cellular concentration of the drug.
- Metabolic alteration and accelerated degradation of the drug.
- Alterations in membrane lipids, which can lead to reduced cellular uptake.
- Alteration of cell cycle checkpoints, inhibition of the repair of damaged DNA and anti-apoptotic defense mechanisms (Kang et al. 2015).

Another problem is that not only cancer cells but also healthy cells, especially rapidly proliferating cells (such as bone marrow, mucosal cells or hair cells), are affected resulting in side effects such as pain, hair loss, nausea, vomiting, fatigue and stomatitis. The extent of the side effects depends on the amount, duration and type of chemotherapy and how the body reacts (National Library of Medicine 2017).
This makes the use of additional drugs for reducing side effects necessary (e.g. anti-emetics, like Ondansetron) (Sandbox Networks 2000b-2017).

3.2.1 Somatic cell gene therapy

Many diseases (often rare diseases) are caused by genetic defects. Also, cancer is counted as a genetic/ immunological disease (American Society of Gene & Cell Therapy 2000-2017).

Genetic defects can be, amongst others, due to:

- point mutation (for example Duchenne muscular dystrophy, an x-linked recessive genetic disorder)
- structural chromosome aberrations like deletion
- chromosomal arrangement (American Society of Gene & Cell Therapy 2000-2017)

Gene therapy focuses on the use of genetic material, which is administered for curing or treating an acquired or inherited disease (American Society of Gene & Cell Therapy 2000-2017). Specific genes are modulated with the intention to reestablish the normal cell equilibrium (American Society of Gene & Cell Therapy 2000-2017).

In the last 28 years (since 1989) gene therapy has changed dramatically and remarkable clinical success could be noted (Keeler et al. 2017). Glybera®, which is based on recombinant adeno-associated virus was approved as the first drug in Europe (Keeler et al. 2017). It is used against lipoprotein lipase deficiency (Keeler et al. 2017). Another example is Strimvelis® (a lentivirus vector) which is used for severe combined immune deficiency (Keeler et al. 2017). Numerous others are in the clinical trial and are still to follow (Keeler et al. 2017). Until End of 2016, almost 2300 clinical trials have been completed in over 3 countries (http://www.abedia.com/wiley/) with more than 60 % of them carried out in USA (http://www.abedia.com/wiley/countries.php). The majority of gene therapy clinical trials have addressed cancer (64.6 %), followed inherited monogenetic diseases (10.5 %) and infectious or cardiovascular disease (7.4 %) (http://www.abedia.com/wiley/indications.php).

Many different gene transfection vectors and delivery techniques have been used (***Figure 2***) (Ginn et al. 2013; Nguyen und Szoka 2012). There are two different groups of vectors, viral and non-viral. In about two- thirds of the clinical trials viral vectors have been used, although

the importance for non-viral vectors increases because of the risk of mutagenesis and induced immune responses against viral carriers (Ginn et al. 2013; Nguyen und Szoka 2012).

- **Viral vectors**

Viruses ("mobile genetic elements") are small obligate intracellular parasites (Gelderblom et al. 1996). They contain either a DNA or RNA (ribonucleic acid) genome which is surrounded by a virus- coded protein coat (Gelderblom et al. 1996). A virus is used as a vector to deliver its genome (DNA or RNA) into the host cell which finally expresses the genetic information of the virus (Gelderblom et al. 1996). Examples for viral vectors used in gene therapy are adenovirus and retrovirus (Keeler et al. 2017). The use of retroviral vectors has decreased in recent years, because of severe adverse events (e.g. unspecific integration may cause activation of oncogenes or inactivation of tumor suppressor genes which can lead to tumorigenesis) (Keeler et al. 2017). Adenoviruses (double-stranded DNA virus) have numerous advantages such as no tumorigenic potential, genetic stability, ability to infect non-dividing cells, high transduction efficiency and transgene expression (Keeler et al. 2017).

Non-viral transfection carriers are of interest because of the oncogenic potential and immunogenicity of viral transfection systems, that leads to an induction of the inflammatory system (Nayerossadat et al. 2012). Another disadvantage of viral carrier is that insertional mutagenesis is possible. Also, their limitation in transgenic capacity size is still a challenge (Nayerossadat et al. 2012).

- **Non-viral vectors (specific, safe and efficient vehicles)**

Numerous non-viral methods (physical and chemical) are used with the aim to transfer genetic information (DNA and messenger RNA, short mRNA) to mammalian cells (Keeler et al. 2017). A variety of these methods have been developed as technologies for gene therapy (in vivo and ex vivo) (Keeler et al. 2017). The most commonly used nucleic acids for gene therapy are modified and unmodified or "naked" DNA molecules (often plasmid DNA - pDNA), small interfering DNA (siRNA) and oligonucleotides (ODN) (Keeler et al. 2017). The spectrum of innovative functional genetic material was broadened and diversified (Zhu und Mahato 2010).

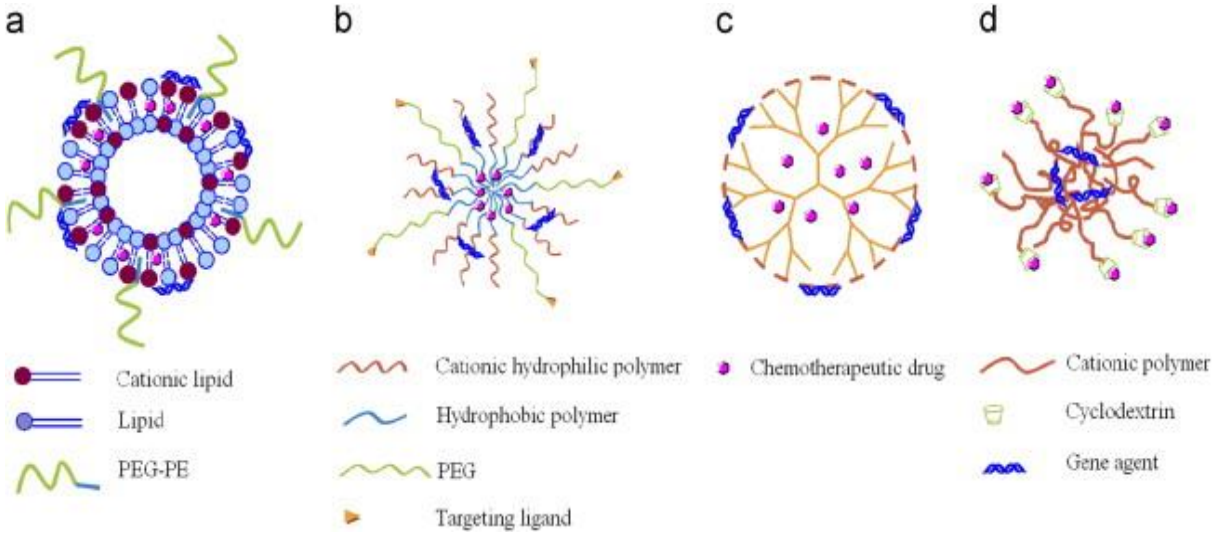


***Figure 2**: major types of nanocarriers: cationic liposomes (a), micelles (b), dendrimers (c) and supramolecular systems (d) (figure from Kang et al., 2015)*

Examples for successful gene transfer are direct intramuscular injection of pDNA (DNA vaccines), intratumoral injection of pDNA (delivery of cytokine and/ or suicide genes), RNA-interference induction, and systemic injection of antisense nucleotides (subcutaneous or intravenous injection leading to induction of RNAse or exon-skipping) (Keeler et al. 2017).

Nevertheless, there are numerous barriers in vivo (after systemic application) that have to be considered in order to reach the target and achieve the expected biological effects (Zhu und Mahato 2010).

In recent years, co-delivery drugs and gene agents have become increasingly important and numerous successes in the treatment of cancer have been noted (Kang et al. 2015). The difficulty is to find efficient carriers. The complexation of nucleic acids with non-viral vehicles is mostly based on electrostatic interaction (Kang et al. 2015; Dai et al. 2015).

- Cationic lipids/liposomes

Cationic liposomes are one of the most common and successfully used drug vehicles for co-delivery in cancer treatment (transfection of nucleic acid) (Kang et al. 2015; Skandrani et al. 2014).

The general structure of a cationic liposome is a cationic head group, a hydrophobic lipid tail and a linker that connects the positive charged head group with the hydrophobic domain (Zhu and Mahato 2010; ***Figure 2 a***).

Cationic liposomes have the ability to bind to the negatively charged nucleic acid (DNA or RNA) and form particles, the so-called lipoplexes that enter cells via adsorptive endocytosis ((Keeler et al. 2017) or related mechanisms. This particle formation results through electrostatic interaction between the cationic lipids and the phosphate backbones of nucleic acids (negatively charged) followed by hydrophobic collapse (Kang et al. 2015; Tsouris et al. 2014).

The structure of the lipid component has a significant influence on transfection efficiency and cytotoxicity of lipoplexes (Zhu and Mahato 2010).

Various technologies of cationic nucleic acid complexes, such as complexes with pDNA, synthetic RNA's, or oligodeoxynucleotides have been developed as potential drugs (Keeler et al. 2017).

An example for a cationic lipid is DOTAP (1, 2- Dioleoyl- 3- trimethylammonium- propane) (Kang et al. 2015; Skandrani et al. 2014).

- Micelle based nanocarrier

Micelle based nanocarriers consist of amphiphilic block copolymers. These co-delivery carriers are biocompatible and physiologically stable because of their low CMC (critical micelle concentration). (Kang et al. 2015; Tsouris et al.2014).

Normally, the micelle self-assembles with the hydrophilic blocks to shape the micelle shell and with the hydrophobic blocks to build the interior, in which poorly soluble hydrophobic drugs can be stored. Hydrophilic blocks, such as Polyethylene Glycol (PEG) or cationic polymers, are used to mask the payloads (Kang et al. 2015; Tsouris et al.2014; ***Figure 2 b***).

- Cationic polymers

Cationic polymers, like Polyethylenimine (PEI) and poly-(2-aminoethyl ethylene phosphate) (PPEEA), build complexes, called polyplexes, with negatively charged nucleic acid (DNA or RNA) (Kang et al. 2015; Tsouris et al.2014). Most commonly used hydrophobic polymers are poly- *ε*- caprolactone (= PCL), polylactide (= PLA), poly- n- butyl acrylate (= PnBA) and poly-lactic-*co*-glycolic acid (= PLGA) (Kang et al. 2015; Tsouris et al.2014). Furthermore, targeting ligands (like folate) may be attached to the outer shell of the carrier to enhance the active targeting-ability (Kang et al. 2015; Tsouris et al.2014).

- Dendrimers

Dendrimers are monodispersed and highly branched macromolecules. Characteristic for these nanocarriers are that they have defined molecular weights and host-guest encapsulation properties (high drug-loading capacity (Kang et al. 2015; Cheng et al 2008).

The general structure of a dendrimer includes (***Figure 3***):

- core
- reactive terminal groups
- branching units , pincer (Verma et al. 2013, Wojciech et al., 2005; Brian and Norman, 2001)

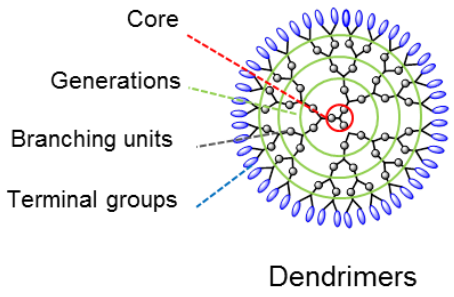


Figure 3 *schematic structure of dendrimers* (dendrimerexpert, 2017)

The interaction with gene and drug molecules takes place via encapsulations, covalent conjugations and electrostatic interactions (Kang et al. 2015; Cheng et al. 2008). A prominent

example for a cationic dendrimer is PAMAM (poly (amidoamine)) (Kang et al. 2015; Esfand et al. 2001).

- supramolecular nanocarrier

Supramolecular nanocarrier represent a complex with a host-guest architecture. The „host" is a chemical compound (most commonly used γ- cyclodextrin) that forms a cavity, into which another chemical compound ("guest") can be placed. However, it is essential that the "host"/"guest" compounds perfectly match (Kang et al. 2015).

3.2.1.1. Polyethylenimine for nucleic acid delivery

Polyethylenimine (PEI) is one of the most efficient non-viral vehicle for nucleic acid delivery acting via polyplexes (Fukumoto et al. 2010; N. A. Nikitenko, V. S. Prassolov 2013). There are two types of PEI, linear Polyethylenimine (LPEI) and branched Polyethylenimine (BPEI). LPEI contains only secondary amines, whereas a branched polymer consists of primary, secondary, and tertiary amines (with a high batch to batch variability (Wightman L. et al. 2001) (***Figure 4***).

a **b**

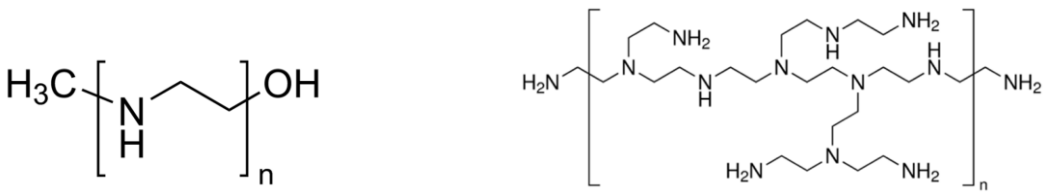


Figure 4*: chemical structure of linear Polyethylenimine (LPEI;* ***Figure 4a****) and branched Polyethylenimine (BPEI;* ***Figure 4b****) taken from Sigma- Aldrich®.*

Further, the N/ P-ratio has an impact on the efficiency of the delivery and toxicity of the PEI (Zhao et al. 2009). The N/ P-ratio represents the ratio between amino groups of the PEI molecule and the phosphate groups of the nucleic acid component in the polyplex (Zhao et al. 2009).

Cationic polymers, such as PEI, can form nanoscale holes into the cell membrane (Hong et al. 2006). This leads to a transient increased permeability of the membrane which is the reason for the high cytotoxicity of PEI (Hong et al. 2006). Polyethylenimine with a low molecular weight (< 2 kDa) is less cytotoxic than a high-molecular- weight PEI (25 kDa; due to a high charge density stronger intensity between PEI and nucleic acids), but it is also characterized by lower transfection efficiency (Breunig et al. 2007). Both in vitro and in vivo, LPEI shows a higher transfection efficiency for pDNA compared to BPEI (Rödl et al. 2013). This is also due to lower binding strength of LPEI to DNA (in Polyplexes) in comparison to branched PEI (Rödl et al. 2013). Therefore, nucleic acid is released more easyly (Rödl et al. 2013).
After i.v. (intravenous) injection into mice, LPEI based polyplexes aggregate due to interaction with blood components, which results in transfection of mainly lung tissue (entrapment in the lung). A considerable amount of it is redistributed to the liver (Rödl et al. 2013, Zintchenko et al., 2009).

The high cytotoxicity as well as its limited biodegradation and the low stability of polyplexes limit the use of Polyethylenimine in clinical applications (Fukumoto et al. 2010).
Thus, further modifications of PEI have been done in recent years (Kichler 2004). The first chemical modification (surface functionalization) to mention is the PEGylation of PEI (Alexis et al. 2008; Kichler 2004). The coupling of a hydrophilic polymer (Polyethylene glycol, short PEG) to PEI reduces protein binding and increases circulation half-life and stability of the generated polyplexes (Alexis et al. 2008; Kichler 2004). A disadvantage of the PEGylation is the reduced cellular uptake (Pozzi et al. 2014) .
A further development of this is the EGFR (epidermal growth factor receptor)- mediated delivery (***Figure 5***) using PEGylated LPEI conjugated with an additional targeting peptide, such as GE11 (***Figure 6***) (Schäfer et al. 2011).

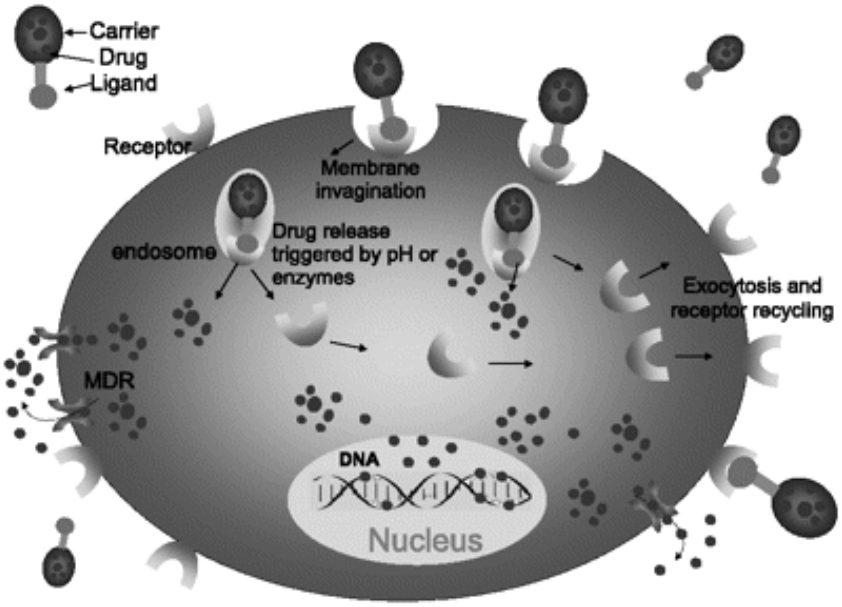


Figure 5: *Schematic representation of receptor – mediated uptake: Nanoparticles conjugated with specific ligands bind to receptors on the cell – surface (in our case Polyplexes with LPEI – PEG – targeting peptides bind on EGFR on the surface of a cell). Thereby the internalization of the nanocarriers into the cell is triggered (through endosome). Due to acidification in the interior of the endosome, the content of the nanoparticles (e.g. drug or in our case pDNA) is released, goes into the cytoplasm and afterwards into the nucleus. The receptors are recycled and migrate back to the cell – surface (figure from Cho et al. 2008).*

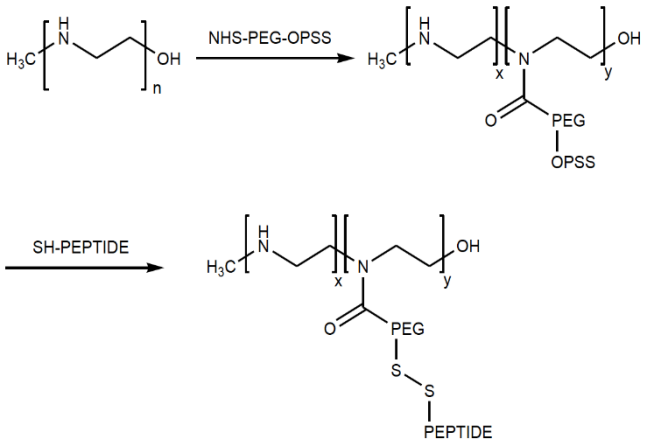


Figure 6: *Scheme of LPEI – conjugates synthesis: A heterobifunctional PEG linker,* ***NHS – PEG – OPSS*** *(3-(2-pyridyldithio) propionamide – PEG – NHS ester) ensures the formation of LPEI – PEG – peptide conjugates.*

EGFR activation caused by the use of such EGFR targeting peptides needs to be avoided because it may cause severe effects concerning the EGFR signaling in cancer (e.g. cell differentiation, proliferation, enhanced migration and adhesion, or apoptosis inhibition of tumor cells (Normanno et al. 2006).

So the aim of targeted polyplexes is a specific EGF-receptor - targeted gene transfer which enhances gene transfection without activating EGFR, such as GE11 polyplexes (Schäfer et al. 2011).

GE11 (sequence: YHWYGYTPQNVI) binds specifically to EGFR. GE11 – conjugated PEI is a safe and efficient vector because it is less mitogenic than EGF (epidermal growth factor) and was found during screening a phage display screening (Li et al. 2005).

Another example for a small peptide used for EGFR – targeting is LARLLT. Besides the EGFR – targeting ability (***Figure 7***, binds in red area), ease of conjugation to other molecules, low immunogenicity makes it an attractive EGFR – targeting biomolecule for selective delivery (Ongarora et al. 2012). LARLLT was found as an EGFR peptide ligand during the screening of a virtual library (Ongarora et al. 2012).

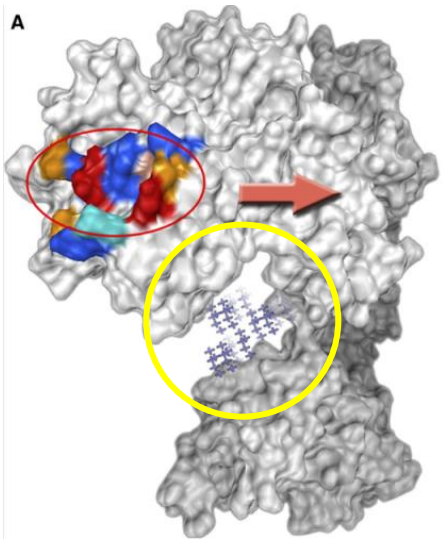


Figure 7: *Cristal structure of EGFR with the binding area (red area) of the EGFR- targeting peptide LARLLT. Yellow encircled area shows the binding site for EGF (not used for targeting). Figure taken from Song S, Liu D, Peng J, et al. Proposed Interaction site of LARLLT with EGFR. FASEB J. 2009;23(5):1396–1404.*

3.3.Mechanism of nucleic acid delivery

Since suitable and safe vectors are needed for optimal delivery of the nucleic acids various lipidic (→ for lipoplexes) and polymeric (→ for polyplexes; such as LPEI) non-viral nanosized vehicles have been developed over the last two decades (Nguyen und Szoka 2012). Unfortunately, none of these non-viral vectors has received the approval of the FDA (U.S. Food and Drug Administration) until now (Nguyen und Szoka 2012; Acton Q. Ashton: Advances in Intracellular Space Research and Application. ScholarlyEditions, Atlanta 2013, page 100). This is due to a low intracellular uptake which leads to a low transfection efficiency (Nguyen und Szoka 2012).

Through binding, complexation or encapsulation of nucleic acid, its delivery into a particular body compartment is possible, avoiding premature degradation (Nguyen und Szoka 2012). Polyplexes (complexes of cationic polymers and nucleic acid) enter cells by adsorptive endocytosis (Nguyen und Szoka 2012).

The internalization of polyplexes depends on their physicochemical characteristics such as size and shape of the generated particles, surface charge, hydrophobicity and complexation conditions (N/P – ratio, concentration, and buffer used for polyplex generation and incubation time) (Lechardeur et al. 2005). Also, the cell internalization of the polyplexes is affected by the cell type (Lechardeur et al. 2005). For cell – specific gene delivery a conjugation of polyplexes with targeting groups is required (specific interactions with target cells) (Ogris et al. 2001).

In adsorptive – mediated endocytosis, polyplexes bind to the cell surface where a charged interaction with complementary binding sites takes place (Khalil et al. 2006). The particles are engulfed and endosomes (membrane – bound vesicles) are formed (Khalil et al. 2006).

After cell uptake, the endosomal escape of polyplexes by proton sponge effect follows (El-Sayed et al. 2009; Nguyen und Szoka, 2012; ***Figure 8***).

Protons are actively pumped into endosomes by membrane bound ATPase. LPEI has a strong pH-buffering effect (pH 5-7)(Nguyen und Szoka 2012; W. Liang and J. K. W. Lam 2012). Due to this strong buffering capacity an acidification within the endosomes can be prevented (Nguyen und Szoka 2012). The accumulation of protons in the endosome triggers a passive chloride influx (El-Sayed et al. 2009; Nguyen und Szoka 2012). Increasing ionic concentration within the endosome leads to water influx to balance the high osmotic pressure (El-Sayed et

al. 2009; Nguyen und Szoka 2012). As a result, osmotic swelling and the rupture of the endosome is promoted and nucleic acids are released into the cytoplasm (El-Sayed et al. 2009; W. Liang and J. K. W. Lam 2012).

Apart from the proton sponge effect another theory has been described, namely the umbrella effect hypothesis (Nguyen and Szoka 2012). This occurs when a polymer (cationic) and a nucleic acid (negatively charged) form a complex and generate small, compressed particles due to electrostatic interactions (Nguyen and Szoka 2012).

After the uptake of polyplexes into the cells and the endosomal acidification follows (Nguyen and Szoka 2012). The polymers are able to unfold and fully expand when the terminal amino groups are protonated at lower pH of 5-6 (Nguyen and Szoka 2012). The reason for this spreading out of neighboring terminal branches is the electrostatic repulsion of the positively charged tertiary amines (Nguyen and Szoka 2012).

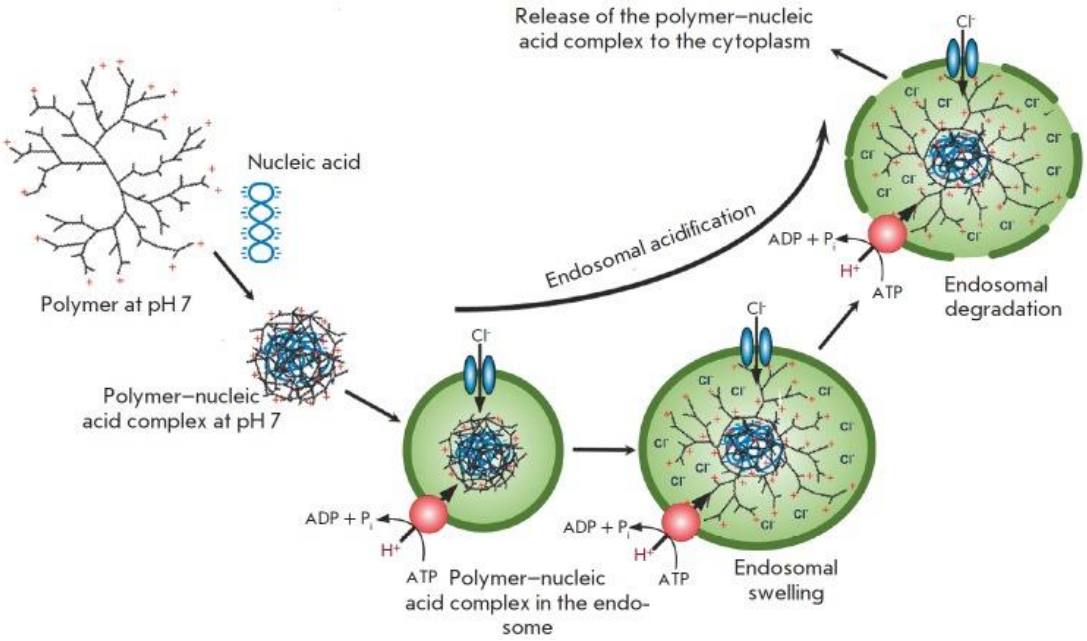
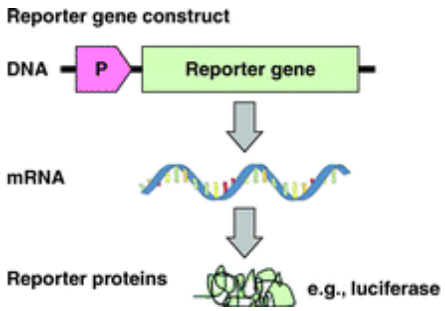


***Figure 8**: Schematic representation of proton sponge effect and umbrella effect hypothesis (Nguyen und Szoka 2012; N. A. Nikitenko, V.S. Prassov 2013).*

3.3.1. Reporter genes

Reporter genes are genes that are used to detect or measure gene expression. Attached to a regulatory sequence or a gene of interest, they can be used for reporting location or level of the expression. Genes, coding for enzymes and fluorescent proteins can be used (https://www.nature.com/subjects/reporter-genes) (***Figure 9***).



p ... promotor
mRNA ... messenger RNA

Figure 9 *(from Youn et al. 2013): Schematic shows promotor-reporter construct in transfected cells. RNA-polymerase and transcription factors can bind on the promotor region and therefore regulate the transcription of reporter gene. A direct correlation between the amount of expressed reporter proteins and the transcriptional activity is given (Youn et al. 2013). The expression of reporter genes can be constitutively or inducible (https://www.thermofisher.com/at/en/home/references/gibco-cell-culture-basics/transfection-basics/reporter-gene-assays.html).*

It is important that the ideal reporter gene has no influence on the physiology of transfected cells and the transgene product in non-cytotoxic. Moreover, it should have a broad linear detection range and should be assayed conveniently.

Examples for such reporter proteins are fluorescent proteins (green fluorescent protein (GFP, jellyfish), red fluorescent protein (RFP, anthozoan) and yellow fluorescent protein (YFP, genetic mutant of GFP)), chloramphenicol acetyltransferase (CAT, bacterial), β – galactosidase (bacterial) and luciferases.

In this work, we focused on using *Gaussia luciferase* as reporter. This reporter luciferase is produced by marine copepod Gaussia princeps (***Figure 10***).

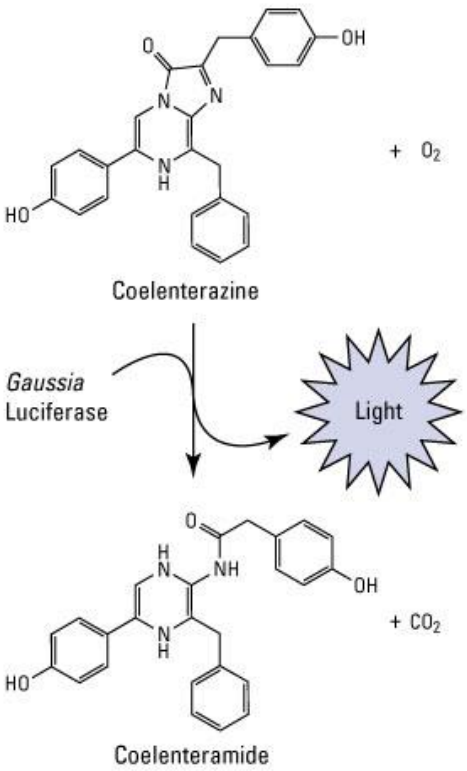


Figure 10: *Gaussia Luciferase catalyses the oxidative decarboxylation of coelenterazin (substrate). Coelenteramide, carbon dioxide and light are produced. The luminescence is measured from the supernatant of cultured cells transfected with a plasmid expressing Gluc is proportional to the amount of enzyme produced. An advantage of Gaussia Luciferase is that it gets secreted and therefore cells can be used for further evaluations like flow cytometry based toxicity assays (https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-assays-analysis/reporter-gene-assays/luciferase-assays/gaussia-luciferase-assays-vectors.html).*

Another example for a luciferase is firefly luciferase which is produced by species of the lampyridae family (beetles). The intracellularly expressed luciferase oxidizes D-luciferin (substrate) to oxyluciferin. A correlation between the bioluminescent signal (light) and the amount of the produced firefly luciferase protein can be done. Thereby, the activity of the promotor, which drives the firefly expression, can be determined (https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-assays-analysis/reporter-gene-assays/luciferase-assays/firefly-luciferase-assays-vectors.html).

4. Aim of the thesis

The overall aim of this diploma thesis was to investigate the suitability of LPEI (10 kDa)- PEG (2 kDa)-CLARLLT, kindly provided to me by Mag. pharm. Taschauer, for EGFR mediated gene delivery performed on human and murine EGFR overexpressing cancer cell lines. Within this, the following aims had to be investigated:

1. characterize cellular morphology, cell growth, apply antibody staining.

2. Optimize transfection assays and evaluate transfection efficiency of CLARLLT targeted polyplexes on the human lung carcinoma cell line A549.wt. cells, CT26.wt murine colon carcinoma cells and LS174T.wt human colon carcinoma cells in a 96-well format.

3. Evaluate toxicity of transfection compounds by flow cytometry based toxicity assays including total cell count after life/dead stain of cells.

4. Evaluate cellular uptake of LPEI- CLARLLT- conjugate by confocal laser scanning microscopy.

5. Materials and Methods

5.1. Chemicals and Reagents

Table 1 *List of chemicals and reagent*

Chemicals	***Supplier***	***Cat.No.***	***Abbreviation***
2-(4-(2-Hydroxyethyl) -1-piperazinyl) -ethansulfonsäure (HEPES)	AppliChem Panreac ITW Companies	A3724,0500	HEPES, $C_8H_{18}N_2O_4S$
4% Formalin in HBS	kindly provided by	Marlene Lutz	
4',6-Diamidin-2-phenylindol (DAPI) 1µg/ml in Milli Pore water	Sigma-Aldrich®	D9542	DAPI
Agarose	Serva®	11404.04	
Bovine Serum Albumin (BSA)	Sigma-Aldrich®	A9647 50g	BSA
D (+) Glucose	Merck KGaA	1.08337.1000	Glucose, $C_6H_{12}O_6$
distilled deionizedUltrapure water (dd water), sterile	obtained using Arium Pro VF ® (Sartorius)		
Dulbecco's Modified Eagle's Medium – high glucose	Sigma-Aldrich®	D5671 500ml	DMEM high glucose
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich®	D8537 1L	DPBS, PBS
Ethylenediaminetetraacetic acid	VWR®	443885J	EDTA
FastRuler™ Middle Range DNA Ladder ready-to-use	Thermo Fisher scientific	SM1113	
Fetal Bovine Serum (FBS)	Sigma-Aldrich®	F7524	FBS
L-Glutamine, 200mM	Sigma-Aldrich®	G7513	
Linear polyethylenimine, Mw= 10 kDa	kindly provided by Mag. pharm. Alexander Taschauer		LPEI 10kDa
Minimum Essential Medium Eagle	Sigma-Aldrich®	M5650	MEME
Opti-MEM® Reduced Serum Medium	Gibco™	11058021	Opti-MEM®, OMEM
pCMV-Gluc	New England Biolabs		
pCpG-hCMV-EF1α-LucSH	characterized by Terese Magnusson (Magnusson, 2011)		
Penicillin/ Streptomycin	Sigma-Aldrich®	P0781	Pen/Strep
RPMI-1640 Medium	Sigma-Aldrich®	R0883-500ml	
Sodium chloride	AppliChem	A2942,5000	NaCl
TrypLE ™ Express (1x) [+] Phenol Red	Gibco™	12605-010 100ml 12605-036 20x 100ml	
Tween 20	Sigma-Aldrich®	P2287-500ml	
Versene 1:5000 (1x)	Gibco™	15040-033 100ml	

5.2. Supplies

Table 2 *List of supplies*

Supply	***Supplier***	***Cat.No.***
Cell culture flask T-75	Cellstar®, Greiner bio-one	REF 658175 LOT E14073BC
cell culture microplate, 96 wells, PS, F-bottom	Sigma-Aldrich®	REF 655098 LOT E 16053 E5
cell culture microplate, 96 wells, V-bottom	Sigma-Aldrich®	651160 LOT E 15063Q6
Centrifuge tube 15 / 50	Star Lab	LOT E 1604334
Cryogenic store vials	Thermo Scientific	374502
Culture flask T-125, 5x	Sarstedt	REF 83.3912.002 LOT 6021511
Culture flask T-25, 10x	Sarstedt	REF 83.3910.002 LOT 6020411
Culture flask T-75, 5x	Sarstedt	REF 83.3911.002 LOT 6022511
Eppendorf tubes	0,5ml nerbe 2,0ml VWR	
Gloves (normal and nitrile)	Starguard	
Laboratory film	Bemis®	PM-999
Lids for 96- microtiter plates, sterile	Sigma-Aldrich®	LOT 2719641
Nunc 1,8 ml external-thread 2D coded universal system cryogenic tubes (48 tubes per bag)	Thermo fisher scientific	LOT 1162177
Parafilm	BEMIS	PM-996
Pipette tips 10µl,200µl,1000µl	nerbe plus	
Reagent reservoir 50ml	VWR®	LOT 124417
Serological pipets 5ml, 10ml, 25ml	BD Falcon	REF 357530 LOT 3276530
Snap-cap	Rotilabo®	18090906
Soft Loops 10µl sterile	nerbe plus	09-421-5065
Syringe filters, cellulose acetat, sterile, 0,22 µm	Rotilabo®	KC 701

5.3.Equipment

- accu-spin™: Fisherbrand
- Analytical balance (Sartorius)
- Arium Pro VF ®: Sartorius (used for creating ddH_2O)
- Biorad PowerPac 200 Electrophoresis Power Supply
- Biorad Wide Mini – Sub Cell® GT
- Centrifuge: Thermo scientific (Heraeus Megafuge 16R centrifuge)
- ChemiDoc XRS + System
- Leica TCS SPE (Confocal Microscope)
    - AC3APO 20x 0.06 IMM objective
- F1-ClipTip™ Multichannel Pipette with 12-channel (10 to 100 µl): Thermo Scientific (Cat. No. 4661170)
- Filter paper (Whatman, Nr. 3)
- FlowJo® (version 10.0.7, 32bit, www.flowjo.com)
- Fluorescence Microscope Leica DMIL LED with a Leica DFC450 C-camera:
    - 10x and 20x lenses from Leica: both air lenses
    - 40x and 63x lenses from Zeiss (apochromatic objective): oil immersion
    - pictures were taken with “LAS X”-software (2.0.0.14332 version)
- Fridge/ Freezer:
    - -150°C: REVCO, Thermo scientific
    - -80°C: REVCO ExF, Thermo scientific
    - -20°C: Allectric (Liebherr)
    - +4°C: Allectric (Liebherr)
- GeneQuant® 1300 UV/Visible cuvette spectrophotometer
- GraphPad Prism 6 and 7 (www.graphpad.com)
- Hemocytometer: Paul Marienfeld GmbH & Co. KG (Germany)
- Heracell™ 150i, CO_2 incubator: Thermo Fisher Scientific™
- Inverted Microscope (S1): AE31 Elite Trinocular, Motic
- Laborboy: accu - jet ® Brand
- Laminar air flow: HERASAFE KS from Thermo scientific
- MACSQuant® Analyzer 10: MACS Miltenyi Biotec (Cat.No.130-096-343)

- Magnetic stirrer: Heidolph Instruments (Cat. No. MR 3001K)
- Micro centrifuge: VWR (Microstar 17R)
- Mr. Frosty Nalgene®: Thermo Scientific (Cat. No. 5100-0001)
- Multi-Channel Ultra High-Performance Pipettor (12-Channel Pipettor; 50- 300 µl): VWR (Cat. No. 89134-758)
- NanoVue® UV/Visible spectrophotometer: GE Healthcare Life Sciences
- pH - meter: WTW
- Plate shaker Eppendorf ThermoMixer®C: Eppendorf (05-412-503)
- Platereader: Infinite® M200 Pro, Tecan
- Thermomixer C: Eppendorf
- Vacuum pump: Integra Vacusafe
- Vortex mixer: VELP scientifica Zx4 (advanced IR vortex mixer)
- Waterbath: VWR (VWB18)

5.4. Buffer, Solutions and Substrates

- ***Preparation of 200ml HBG (HEPES buffered glucose buffer)***

10g Glucose and 953.2 mg HEPES (Mw: 238.31g/mol) were dissolved in 150ml ddH2O. The pH was adjusted to 7.40 with 1 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH). The solution was filled up to 200 ml with ddH2O, passed through a 0.22µm filter and stored at +4 °C.

- ***Preparation of 200ml HBS (HEPES buffered saline buffer)***

20mM HEPES and 150mM NaCl were filled up to 200ml with ddH_2O.

1.753g NaCl and 953.2 mg HEPES (Mw: 238.31g/mol) were dissolved in 150ml ddH_2O. The pH was adjusted to 7.40 with 1 M HCl and 1 M NaOH. The solution was filled up to 200 ml with ddH2O, passed through a 0.22µm filter and stored at +4 °C.

- ***Buffers used for intracellular antibody staining***
    - **permeabilization buffer**

      250 µl Tween 20 was added to 49.75 ml DPBS.
    - **washing buffer**

      5 µl Tween 20 was added to 50.00 ml DPBS.
    - **antibody buffer**

      250 µl Tween 20 and 1g BSA (bovine serum albumin) were added to 49.75 ml DPBS.
    - **blocking buffer = PEB**

      250mg BSA and 29.23mg EDTA (Ethylenediaminetetraacetic acid) were added to 50.00 ml DPBS.

- ***Coelenterazine (CTZ) reconstitution- buffer***

Sodium chloride (NaCl) was dissolved in DPBS at a concentration of 5 mM. The solution was passed through a 0.22 µm filter.

- ***Preparation of Coelenterazine (CTZ) substrate for Gaussia luciferase assays***

CTZ (5 mg/ml in dry DMSO) was further diluted with CTZ reconstitution buffer to a final concentration of 1.694 µg/ml. Due to autoxidation CTZ solution should equilibrate for 30 min at room temperature until further usage. For *Gaussia luciferase* (GLuc) assays CTZ solution was always prepared freshly.

- ***Preparation 4% (w/V) formaldehyde in HBS pH 7.4 (for 2L)***

80g paraformaldehyde was added to 500ml HBS and heated at 70°C with a magnetic stirrer until complete dissolution. The pH was adjusted to 7.4 with 1 M HCl and 1 M NaOH and the solution was filled up to 2l with HBS.
The solution was filtered through a folded filter paper and stored at +4°C.

- ***1l 20X SB- buffer (sodium borate), self-made***

8g NaOH and 48 g boric acid were dissolved in 800 ml ddH_2O. The pH was adjusted to 8 using 6 M HCl and 10 M NaOH. The solution was filled up to 1000 ml with ddH_2O and autoclaved.

5.5. Plasmid DNA quality analysis

Miniprep and Gigaprep (NucleoBond PC10000 GigaPrep from Macherey Nagel) were conducted as per manufacturer's instructions.

As quality controls both pDNA quantification by UV/Vis and a diagnostic restriction digest were conducted. The restriction digest of pCMV-Gluc (a plasmid that expresses the secreted *Gaussia luciferase* constitutively under the control of the cytomegalovirus (CMV) promotor) was carried out with FastDigest® restriction enzymes (HindIII, HindIII + XhoI and SalI + ScaI).

5.6. Quality control of generated polyplexes

Since we wanted to use polyplexes for our transfection assays, we had to check biophysical properties of generated polyplexes. The polyplexes were generated by flash pipetting. The quality of generated particles was analyzed by NTA (nanoparticle tracking analysis) based on the protocol described by Taschauer et al. 2016.

Two different polyplexes were tested: **pCpG-hCMV-EF1α-LucSH/ LPEI 10 kDa (N/P 6)** and **pCMV-Gluc/ LPEI 10 kDa (N/P 6)**.
Size and polydispersity of our generated particles was as per literature (Wightman et al. 2001; results and ***Figure 14*** in section 6.2.).

5.7. Antibody staining in 96 - well plates – EGFR expression

5.7.1. EGFR antibody staining (direct labeling)

Materials:

- ***reagents:***
  - PEB (blocking solution; prepared as described in 5.4.)
    - Antibodies were diluted in PEB
      - Antibody: Mouse IgG 1, kappa (Alexa Fluor® 488 anti-human EGFR)
      - Isotype ctrl: Mouse IgG1, kappa (Alexa Fluor® 488)
    - UT (untreated cells): pure PEB
  - appropriate complete culture medium with supplements (everything from Sigma-Aldrich®; prepared as per manufacturer's instructions)
    - Versene 1:5000 (1X)
      =0.5 mM EDTA in Phosphate buffer, which complexes Ca^{2+} ions that are needed for cell attachment. It was used for cell detachment in order not to digest antigens on cellular surface.
- ***samples:***
  - A549.wt or LS174T.wt cells
- ***supplies:***
  - V-bottom - well plate, CT - 50 and aluminum foil

Cell preparation and cell staining:

First, the medium in the cell culture flask was removed, the cells were washed with PEB, detached with Versene and put into the incubator (37°) for 5 minutes. Afterwards, they were resuspended in PEB.

Cells were counted by flow cytometry. 0.2 x 10^6 cells per well were used for antibody staining in a V-bottom plate.

The cell suspension was centrifuged at 200 g for 5 minutes at room temperature. Then, the supernatant was aspirated, the cells were resuspended in 100 µl PEB. Then, the plate was centrifuged (in a plate rotor) at 600 g for 5 minutes.

The supernatant was aspirated and **20 µl** of the antibody solutions was added per well (working with light protection!). Samples were incubated for 30 minutes at 4°C in the dark. After the incubation, the cells were centrifuged down at 600g for 5 minutes at 4°C. The supernatant was aspirated and the cell pellet resuspended in 100µl cold PEB. PEB wash was done twice.

The measurement was performed by flow cytometry with a MACSQuant® Analyzer 10. The obtained data was analyzed by using FlowJo™ and visualized with Excel and GraphPad Prism 6 and 7 (www.graphpad.com).

5.7.2. Intracellular antibody staining (indirect labeling) of CT26.wt

Materials:

- ***reagents:***
  - PEB (blocking solution; prepared as described in section 5.4.)
  - permeabilization buffer, washing buffer and antibody buffer (prepared as described in section 5.4.)
  - appropriate complete cell culture medium (prepared as per manufacturer's instructions)
  - Versene 1:5000 (1X)
  - Antibodies were diluted in PEB
    - primary antibody: Rabbit IgG Anti-mouse EGFR for intracellular EGFR staining *[ab52894; EP38Y; GR41670; Abcam]*
    - secondary antibody: Goat Anti-Rabbit IgG H&L (Alexa Fluor ® 488) *[ab150077; GR149012-1; Abcam]*
    - Isotype ctrl: Rabbit IgG monoclonal *[EPR25A; Abcam]*
  - UT (untreated cells): PEB (with Tween)

- ***samples:***
  - CT26.wt cells
  - DAPI (1µg/ml)

- ***supplies:***
  - V – bottom 96 - well plate, CT - 15 tubes, FACS – tubes and aluminum foil

Cell preparation and cell staining:

First, the medium in the cell culture flask was removed, the cells were washed with PEB, detached with Versene and put into the incubator (37°) for 5 minutes. Afterwards, the cells were resuspended in PEB.

Cells were counted by flow cytometry and 0.2 x 10^6 cells per well were used for antibody staining in a V-bottom plate.

Then, the plate was centrifuged (in a plate rotor) 200 g for 5 minutes (4°C). The supernatant was aspirated carefully. For permeabilization, cells were resuspended in **70µl permeabilization buffer** (0.5% Tween in DPBS) and incubated in the dark for 15 minutes (at room temperature).

After centrifugation at 200 g (5 min) supernatant was aspirated and the **primary antibody or controls (unstained)** were added.

Samples were incubated for 30 minutes at 4°C in the fridge. Then, the cells were centrifuged at 200g for 5 minutes at 4°C. The supernatant was aspirated and the cells were washed twice with **washing buffer** (0.1%Tween 20 in DPBS). From this moment, it was necessary to **work under light protection**!

The secondary antibody was added and cells were incubated for **30 minutes at 4°C** (plate wrapped in aluminium foil).

After incubation, cells were centrifuged at 200g for 5 minutes at 4°C. The supernatant was aspirated and the cells were washed twice with **washing buffer** (0.1%Tween 20 in DPBS). Then, it was diluted with PEB.

Samples were analysed by flow cytometer with MACS Quant® Analyzer 10. The analysis of the results was done with FLOW JO®.

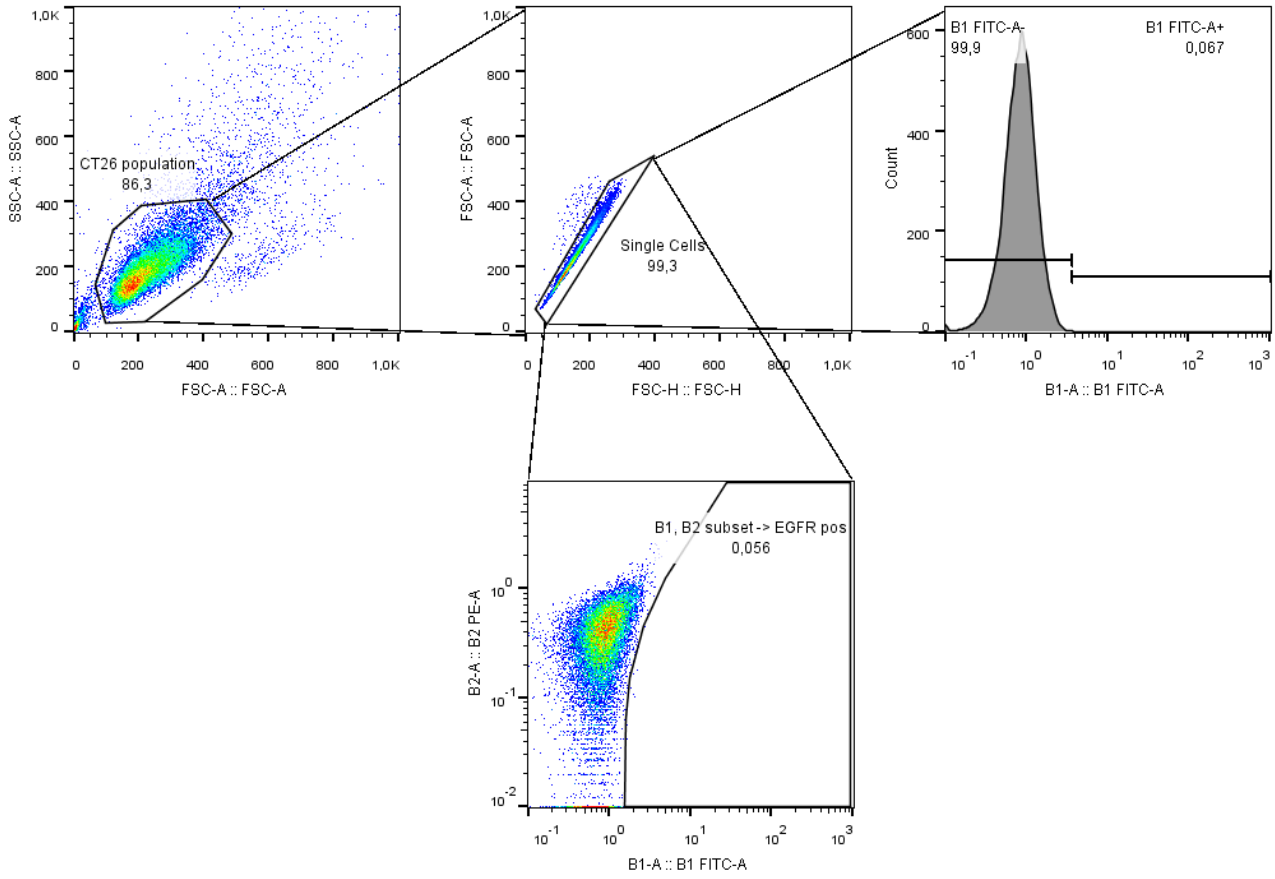


Figure 11: *Gating principle used for intracellular EGFR antibody staining of CT26.wt. Cells were stained with Isotype control. In the first gate (abscissa: FSC-A, ordinate: SSC-A) the cell population was gated (**Figure 11, left**). In the second gate (abscissa: FSC-H, ordinate: FSC-A) single cells were gated (**Figure 11, middle, top row**). In the third gate (abscissa: B1-A, ordinate: count) EGFP positive cells were gated and EGFP negative cells were excluded (**Figure 11, right**). The fourth gate shows another gating type (density plot) for gating the EGFP postive events (**Figure 11, bottom row**). Both histogram (B1 channel) and density plot (abscissa: B1-A, ordinate: B2-A) were used for analysis.*

5.8. Testing of toxicity and transfection efficiency of LPEI-10kDa on A549.wt

For in vitro testing of toxicity and for transfection assays, cells (A549.wt (CCL-185™, ATCC®), 10,000 cells/ well) were counted with the Haemocytometer and seeded in a F-bottom 96 - well plate as described in more detail in *3.6.4.1*. 24 hours before transfection. Each testing was performed in triplicates (2 experiments). Wells with samples were compared to untreated cells (UT).

- Toxicity assay:

The toxicity of: 100ng; 250ng; 350ng; 500ng; 750ng; 1µg; 3µg and 5µg of LPEI – 10kDa (LPEI-concentration/well) in HBG was tested.

After the cell seeding, cells were incubated for 24 hours under standard conditions.

To generate small, colloidal stable polyplexes, the polyplex formation is carried out in a sterile low-salt buffer, such as HBG buffer (20 mM Hepes; 5 % (w/V) glucose; pH= 7.4); preparation see 3.4.) in our case HBG buffer (Rödl, 2013).

Calculations for polyplexes:

200 ng pDNA/well was used for the 96 - well plate experiments and the final plasmid concentration was 20µg/ml.

For calculating the needed amount of LPEI 10kDa to generate polyplexes with certain N/P ratios the following formula was used (Rödl, 2013):

$$m(\text{LPEI}) = 43 \cdot N/P \cdot \frac{m(\text{pDNA})}{330}$$

43 represents the molecular weight of a standard LPEI monomer subunit (MW= 43 g/mol). For N/P ratios 6 and 9 are used. The standard mass of plasmid DNA m (pDNA) is 200 ng and 330 represents the average molecular weight of a nucleotide.

Twentyfour hours after seeding followed by incubation at standard conditions (37 °C, 5 % CO_2), the 96 - well plate was processed as follows: First, medium was aspirated and replaced with 100µl basal medium (pure medium, RPMI-1640 without supplements; Sigma-Aldrich®) and samples, LPEI 10 (for toxicity assay) or polyplexes (for transfection assay) were added. The polyplex solution was generated by **flash pipetting** (method described by Taschauer et al. 2016).

After 4 hours incubation at standard conditions, 100µl complete medium (RPMI-1640 with supplements including 10 % FBS, everything from Sigma-Aldrich®) was added.
After 24 h total treatment time, supernatant was aspirated and the cells were washed once with DPBS (Sigma-Aldrich®). TrypLE ™ (Cat. No. 12605010, Gibco™) was added and cells were incubated for 5 minutes under standard conditions.

Cells were resuspended in DPBS and analysed by flow cytometry. DAPI was used for live/dead staining at a concentration of 1 µg/ml. V1 channel was set based on cellular autofluorescence.

Analysis of results was done with FLOW JO®.

5.9.LPEI- based targeting assays

All experiments were performed in transparent F-bottom 96 - well plates. 10,000 cells/ well (A549.wt, CT26.wt or LS74T.wt) were used for the tests (degree of confluence 70-90%).

5.9.1. Optimization of the transient transfection assay based on A549.wt

The testing was carried out under different conditions:

- ***transfection medium:***
  - basal medium (pure medium without supplements)
  - complete medium (basal medium with supplements: 1% Penicillin/Streptomycin, 10% FBS and L-Glutamine amount based on Sigma® reference)
  - Opti-MEM®
- ***buffer***
  - HBG (20mM HEPES, 5 % (w/V) glucose, pH=7.4); preparation see 3.4.
  - HBS (20mM HEPES, 5 % (w/V) glucose, pH=7.4); preparation see 3.4.
- ***N/P ratios***
  - N/P ratio 6
  - N/P ratio 9
- ***incubation times***
  - 4 hours
  - 24 hours

additional material:

- CTZ reagent (described in section 3.4.)
- DPBS (Sigma-Aldrich) and TrypLE ™ Express (1X) (Cat. No. 12605010, Gibco™)

10,000 cells/ well (A549.wt; ATCC® CCL-185™) were counted and seeded in 200µl of complete medium in a 96 - well plate (preparation see 3.9.4.). Samples were tested in

triplicates. As a negative control, a triplicate of untreated cells (UT; only with complete medium incubated, no sample was added) was taken.
The required volumes of each stock for the generation of polyplexes were calculated (200 ng pDNA/well; see 3.10.).

After 24 hours incubation under standard conditions, the medium was aspirated and replaced with 100µl of basal medium (RPMI-1640; Cat. No. R0883, Sigma-Aldrich®) or complete medium (see) or Opti-MEM® (Reduced Serum Medium; Gibco™) containing the fresh via flash pipetting generated polyplex solution (pCMV Gluc and LPEI-10kDa in HBG or HBS). After 4 hours of incubation, 100µl of complete medium was added cells with 24 hours transfection. For cells with 4 hours transfection time, the medium was replaced with fresh, pre-warmed complete medium (200µl/well).
The cells were incubated for another 20 hours followed by Flow cytometry and *Gaussia luciferase assay* of supernatant (luminescence measurement; with CTZ reagent). DAPI was used for live/dead staining at a concentration of 1 µg/ml.

Flow cytometry was performed with MACS Quant® Analyzer 10 as described in detail in 5.8. The analysis of the results was done with FLOW JO®.

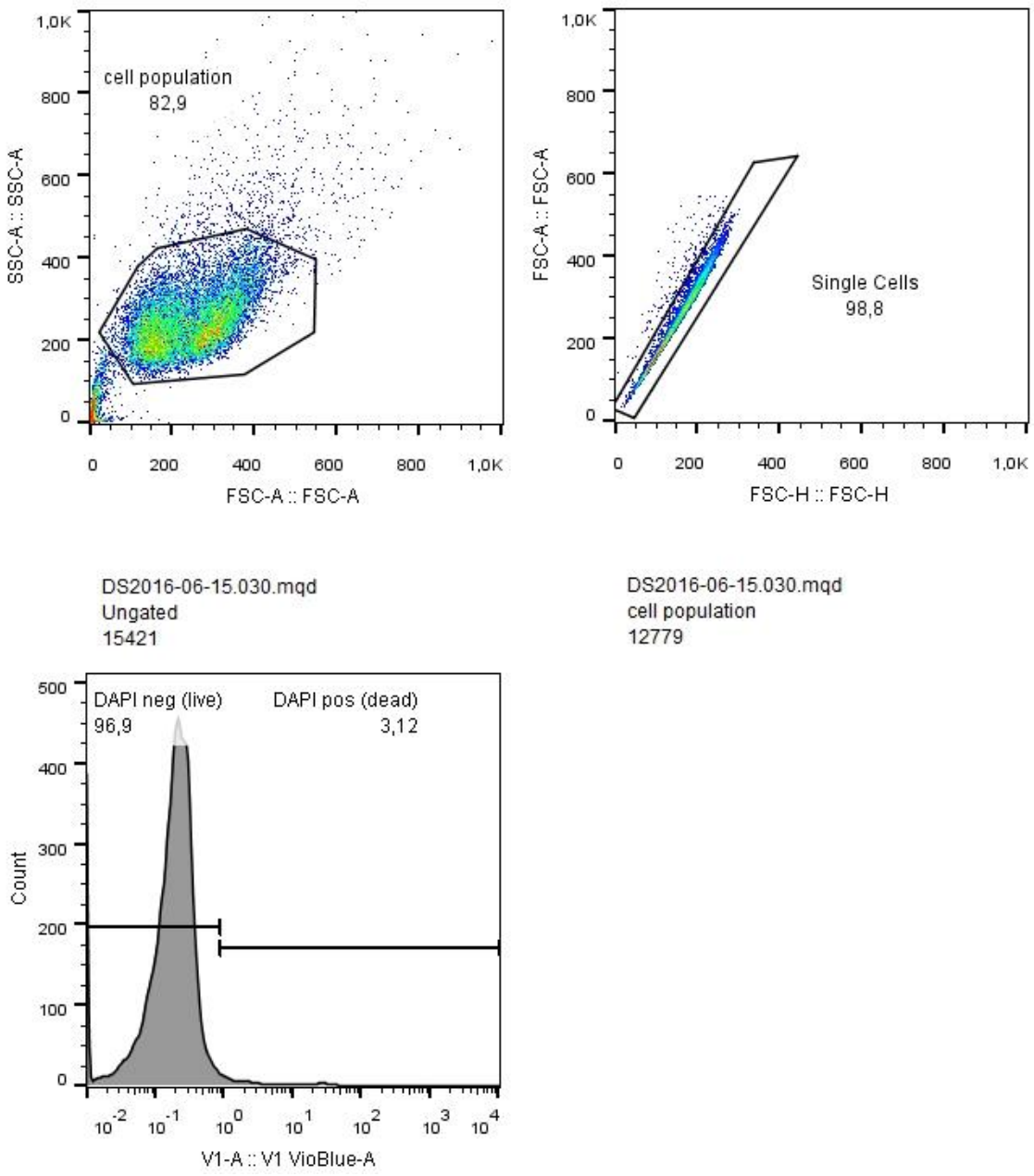


Figure 12: *Gating principle used for live/dead evaluation of A549.wt after treatment. This well was treated with LPEI 10kDa, N/P ratio 9, in HBG with 4 hours incubation in Opti-MEM® used as a transfection medium. In the first gate (abscissa: FSC-A, ordinate: SSC-A) the cell population was gated (**Figure 12, left, top row**). In the second gate (abscissa: FSC-H, ordinate: FSC-A) single cells were gated (**Figure 12, right, top row**). In the third gate (abscissa: V1-A, ordinate: count) living cells (= DAPI-negative cells) were gated and dead cells (=DAPI-positive cells) excluded (**Figure 12, left, bottom row**).*

Gaussia luciferase assay

After 24 hours of transfection the luciferase activity was measured and quantified (relative light unit, RLU's) with the plate reader (Infinite® M200 Pro, Tecan).

For the *Gaussia luciferase* assay 20 µl of the supernatant of each sample is transferred into a white 96-well plate.
Luminescence was measured using the following settings of the plate reader:

1. Volume of CTZ reagent (added by autoinjector): 50 µl
2. Integration time: 10,000 ms
3. Waiting time: 2 sec.

Analysis of results was done with Microsoft Excel® and GraphPad Prism® 7 (www.graphpad.com).

5.9.2 EGFR - Targeting assays with A549.wt, CT26.wt and LS174T.wt cells

10,000 cells/well (A549.wt, CT26.wt and LS174T.wt) were seeded in a transparent 96 - well plate (in section 8.3.3.1.). Each sample was tested in triplicates and as a negative control UT-cells (without a sample, only incubated with complete medium) were used. Polyplexes were prepared at N/P 9 in HBG.

Targeting assays were performed using conditions described in 5.9.1. with LPEI 10 kDa, LPEI 10- PEG2- Cys, LPEI 10- PEG2- GE11 and LPEI 10- PEG2- CLARLLT as components.

5.10. Cell fixation – Nunc chamber optimization with A549.wt cells

The aim of this experiments was establishing a cell fixation protocol for Nunc chamber slides. Different antibody concentrations (0.1µl or 0.2µl) and timepoints (30 or 40 minutes) were tested.

materials	
cells	A549.wt (ATCC® CCL-185™, LGC Standards GmbH from Germany)
medium	RPMI-1640 complete medium (R0883, Sigma-Aldrich®)
antibody	Mouse IgG 1, kappa (Alexa Fluor® 488 anti-human EGFR)
staining- solution	DAPI (1µl/ml, Cat. No. D9542, Sigma-Aldrich®)
blocking- solution	PEB: DPBS with 2mM EDTA and 0.5% BSA,0.22 µm filtered
washing- solution	DPBS (Dulbecco's Phosphate Buffered Saline, Cat. No. D8537, Sigma-Aldrich®)
fixating agent	4% Formalin in HBS (kindly provided by Marlene Lutz)
mounting medium	Vectashield

Table 3 *materials for cell fixation (Nunc chamber slides)*

20,000 cells (A549.wt, ATCC® CCL-185™) suspended in 400µl complete medium were seeded per chamber and incubated for 24 hours.

Thereafter, the Nunc chamber fixation with 4% Formalin as a fixating agent was performed. The experiment was started with the 40 minutes timepoint followed by the 30 minute timepoint. Supernatant was removed and the cells were washed 3 times with DPBS. After that, 200µl 4% Formalin was pipetted into the chambers. Then, cells were washed again 3 times with DPBS, blocked with 400µl PEB buffer per chamber and incubated for 30 minutes at room temperature.

After this, the supernatant was aspirated and antibody staining was performed with extracellular anti-human EGFR antibody (Alexa Fluor® 488; 0.1µl and 0.2µl). Cells were incubated for 30 minutes at room temperature.

Then, supernatant was aspirated, the cells were washed again 3 times with DPBS and 400µl DAPI (1µg/ml in ddH_2O, Cat. No. D9542, Sigma-Aldrich®) was added per chamber and incubated for 10 minutes at room temperature.
The supernatant was aspirated and the cells were washed again 3 times with DPBS.

Afterwards, samples were mounted using 1 drop mounting medium per chamber area.

5.11. Evaluation of uptake via Confocal Microscopy with A549.wt cells

The aim of this experiment was testing of cell binding/internalization of LPEI 10- PEG2- CLARLLT (heptapeptide) on A549.wt cells. The cell binding/uptake of LPEI 10kDa/LPEI 10- PEG2- Cys/ LPEI-PEG2- CLARLLT was analysed and compared.

Cy5 labeled pDNA (pCMV-Gluc) was used.

materials	
cells	A549.wt (CCL-185™, ATCC®) with a confluency of 100%
complete medium	RPMI-1640 (Cat. No. R0883, Sigma-Aldrich®) supplemented with 10% FBS, 3,4ml L-Glutamine and 1% P/S
washing solution	DPBS (Dulbecco's Phosphate Buffered Saline, pH= 7.2-7.4 Cat. No. D8537, Sigma-Aldrich®)
trypsinization reagent	TrypLE™ Express (1X) (Cat. No. 12605010, Gibco™)
buffer	HBG (20 mM Hepes; 5 % (w/V) glucose; pH= 7.4); see at 3.4
fixating agent	Formalin 4% (kindly provided by Marlene Lutz)
mounting medium	Vectashield
compounds for polyplexes (N/P 9) (kindly provided by Mag. A. Taschauer)	LPEI 10 kDa, diluted in HBG - non-targeted (used as control substance)
	LPEI 10- PEG2- Cys - non-targeted (used as control substance)
	LPEI 10- PEG2- CLARLLT - EGFR-targeted
	plasmid DNA: Cy5 labeled pCMV-Gluc

Table 4 *materials for evaluation of uptake via Confocal Microscopy*

20,000 cells/ chamber were seeded in 200µl complete medium in a Nunc chamber slide and incubated for 24 hours.

Then, medium was aspirated and replaced with basal or complete medium. Thereafter, polyplex solution was added (with **400ng pDNA/chamber**) and the cells were incubated for 4 hours. After the incubation, cells were washed twice with DPBS to remove loosely bound polyplexes.

200µl Formalin 4% was added as a fixating agent, cells were incubated for 30 minutes and afterwards 3 times washed with DPBS.

DAPI (1µg/ml) was added for the staining, the cells were incubated for 10 minutes and afterwards 3 times washed with DPBS.

Afterwards, the Nunc chamber slide samples was fixed and 1 drop of the mounting medium was added per chamber were mounted using 1 drop mounting medium per chamber area. Samples were imaged by confocal microscopy at 20X and 40X magnification.

6. Results

6.1. Evaluation of pCMV-Gluc quality

pCMV-Gluc was used as a plasmid with a concentration of 250 µg/ml.

As quality controls both pDNA quantification by UV/Vis and a diagnostic restriction digest were conducted (described in 5.5). For restriction digestion of pDNA sample FastDigest® enzymes (HindIII, HindIII+XhoI and SalI+ScaI) were used. FastRuler® Middle Range DNA Ladder was used as per manufacturer´s instructions. SYBR® Safe was added to the gel as DNA stain and imaging of gel was conducted with a BioRad gel documentation system (***Figure 13***).

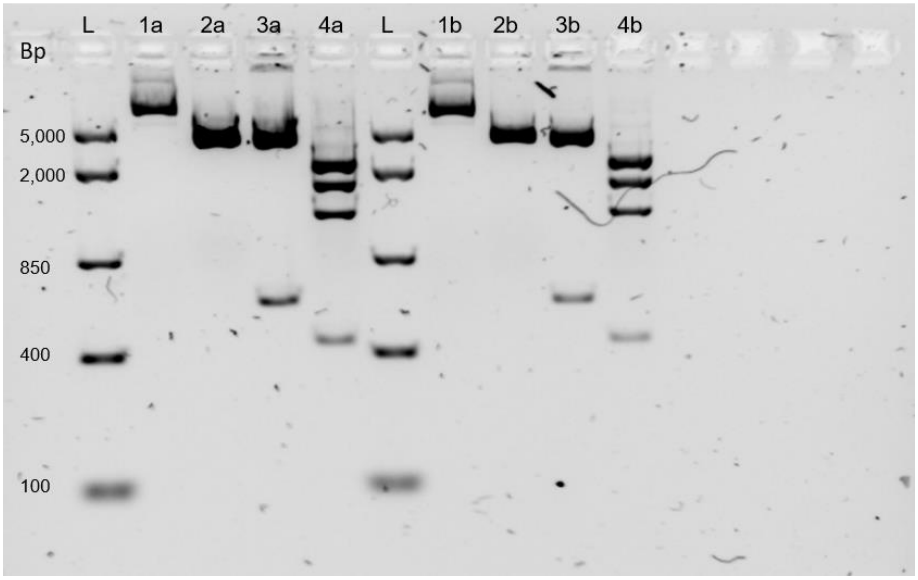


Figure 13 *restriction digest (1% agarose gel with plasmid pCMV-Gluc)*

Table 5*: Sample list used for Figure 13*

sample abbreviation	**L**	**1a**	**2a**	**3a**	**4a**	**L**	**1b**	**2b**	**3b**	**4b**
substance	DNA Ladder	undigested pDNA	HindIII	HindIII + XhoI	SalI + ScaI	DNA Ladder	undigested pDNA	HindIII	HindIII + XhoI	SalI + ScaI
volume	4µl	10µl	10µl	10µl	10µl	4µl	10µl	10µl	10µl	10µl

Each reaction was loaded twice (***Table 5***). Based on "ApE-A plasmid editor" software the theoretical fragment sizes are as follows:

- undigested pCMV-Gluc (sample 1a and 1b): 5764 bp (base pairs)
- pCMV-Gluc + HindIII (sample 2a and 2b): 5764 bp

- pCMV-Gluc + XhoI + HindIII (sample 3a and 3b): 5168 bp; 596 bp
- pCMV-Gluc + SalI + ScaI (sample 4a and 4b): 2318 bp; 1745 bp; 1224 bp; 443 bp; 34 bp

Both undigested pDNA as well as pDNA fragments after restriction digestion show a size that conform to theoretical sizes calculated by “ApE-A plasmid editor” software.

In addition, two approaches were done with the NanoVue® UV/Visible spectrophotometer (blank was ddH_20; 1:5 dilutions):

- 357.0 µg/ml
- 361.0 µg/ml

These two values result in an average pDNA concentration of 1725 µg/ml.

The diagnostic restriction digest in combination with the result of UV/Vis measurements shows a high quality of pDNA sample (pCMV-Gluc). Therefore, this pDNA sample could be used for further experiments (e.g. transfection studies).

6.2. NTA- analysis: quality control of generated polyplexes

A representative polyplex sample was generated and tested by NTA (nanoparticle tracking analysis) to evaluate the quality of polyplex generation protocol and as part of the training. Particles were synthesized using the protocol published by Taschauer et al. The following components were used:

1. linear Polyethylenimine (10 kDa)
2. pDNA: pCMV-Gluc

Polyplexes were generated at N/P 6 in HBG by flash pipetting at a pDNA concentration of 20 µg/ml, further diluted in HBG (dilution factor: 1/50, based on the protocol described by Taschauer et al.) and measured by NTA.

NTA measurement showed the following physical polyplex properties:

1. Mean: 247 nm
2. Mode: 180 nm
3. SD: 73 nm

Size distribution is depicted in ***Figure 14***.

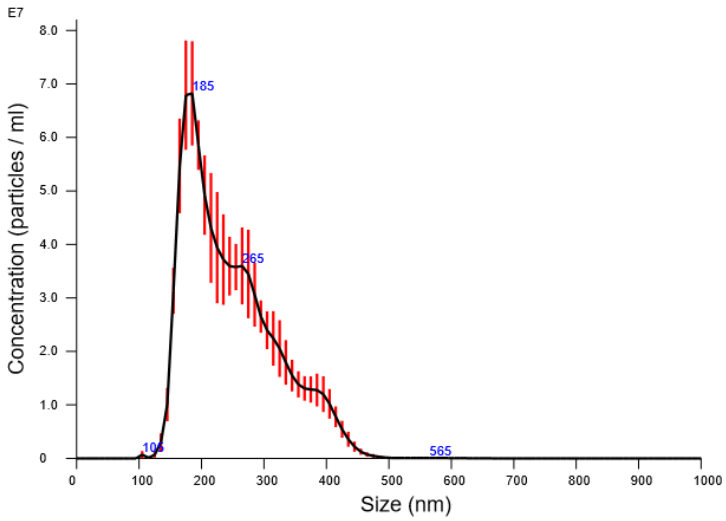


Figure 14 *Example for presentation of a NTA measurement (pCMV-Gluc/ LPEI 10 kDa (N/P 6).*

NTA evaluation shows particle properties as also described in the literature (Wightman et al. 2001). Therefore, protocol for flash pipetting generation can be used for further experiments.

6.3. Antibody staining and evaluation by flow cytometry

6.3.1. Extracellular EGFR antibody staining of human cancer cell lines (direct labeling)

EGFR expression was tested on A549.wt and LS174T.wt cells. 0.2x 10^6 cells per well were used for evaluation of EGFR expression level (in a transparent 96 – well plate with a V – bottom plate) and were treated with:

- Antibody: Mouse IgG 1, kappa (Alexa Fluor® 488 anti-human EGFR) or
- Isotype: Mouse IgG1, kappa (Alexa Fluor® 488) or
- UT: pure PEB (PBS + 5 mM EDTA + 0.5% BSA; untreated)

Antibody staining protocol is described in section 5.7.1. EGFR staining on human cell lines (A549.wt and LS174T.wt) is shown in ***Figure 15***. Subsequent measurement by flow cytometry are described in ***Figures 15***. Both cell lines show high expression levels of EGFR (compared to isotype control two log-shifts on A549.wt and 1 log-shift on LS174T.wt). Both cell can therefore be used for transfection studies based on EGFR mediated cell uptake.

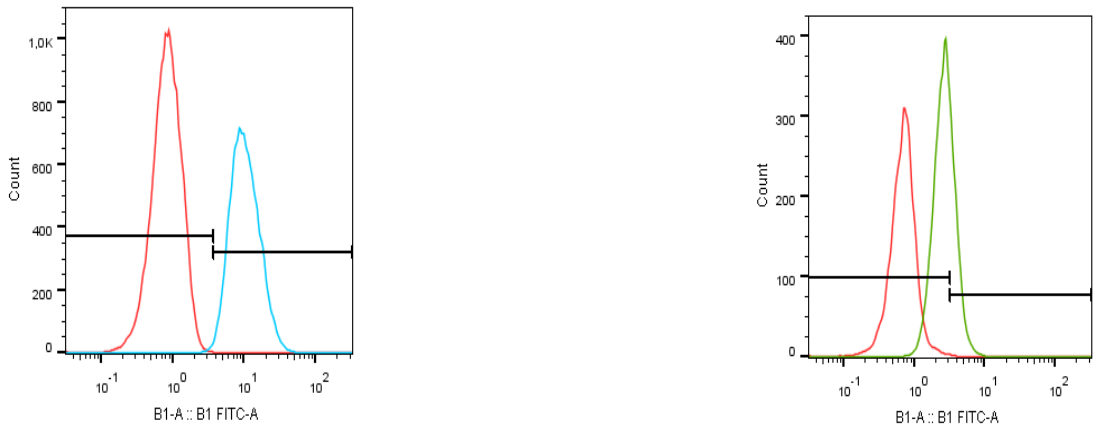


Figure 15*: Representative examples of EGFR expression levels in A549.wt and LS174T. Gating is based on isotype control (red graph both in* ***Figure 15 left*** *and* ***15 right****).* ***Figure 15 left*** *shows EGFR expression level of A549.wt (blue histogram).* ***Figure 15 right*** *shows EGFR expression level of LS174T.wt (green histogram). Both cell lines showed at least a one log shift between isotype control and EGFR positive events (A549.wt cells 2 – log A549.wt cells 2 log-shifts; LS174T.wt cells 1 log-shift).*

6.3.2. Intracellular EGFR antibody staining of murine cancer cells (indirect labeling)

Evaluation of EGFR expression was carried out on CT26.wt cells. were used for evaluation of EGFR expression level (in a transparent 96 – well V – bottom plate) and were treated with:

- Antibody mixes:
  - Isotype ctrl: Rabbit IgG monoclonal
  - primary Ab: Rabbit IgG Anti-mouse EGFR for intracellular EGFR staining and secondary Ab: Goat Anti-Rabbit IgG H&L (Alexa Fluor ® 488)
- UT: PEB (with Tween)

The working steps of that antibody staining are described in detail in section 5.7.2. The subsequent measurement by flow cytometry are described in ***Figure 16***. Based on evaluation of flow cytometry measurements CT26.wt cells show medium-low EGFR expression (20-30% positive events) and were therefore further used for transfection studies based on EGFR mediated cell uptake.

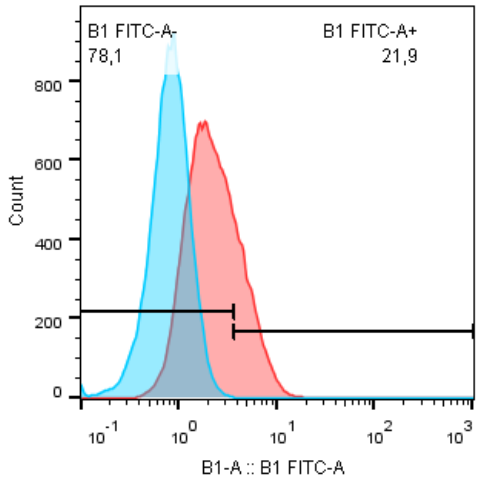


Figure 16: *Representative examples of EGFR expression level in CT26.wt. Gating is based on isotype control (blue histogram). EGFR – positive events are presented as a red histogram.*

6.4. Testing of cytotoxicity of LPEI (10kDa) and LPEI (10 kDa) based polyplexes on A549.wt

6.4.1. Effect of dose modification and N/P ratio on cytotoxicity

A549.wt cells (CCL-185™, ATCC®, LGC Standards GmbH from Germany) were treated with different concentrations of LPEI (10 kDa) and polyplexes based on pCMV-Gluc and LPEI (10 kD) prepared at N/P 6 and 9. 1x 10^4 cells per well were seeded into a 96 – well plate (transparent; F-bottom). LPEI-10 kDa was tested at concentrations ranging from 100 ng/well to 1000 ng/well. Polyplexes with N/P ratios of 6 or 9 were tested. Polyplexes were prepared at a pDNA conc. of 20 µg/ml in HBG by flash pipetting. Polyplex treatment of cells was conducted with a pDNA concentration of 2 µg/ml. All samples were tested in triplicates. The testing was performed as described in section 5.8.

Figure 17 shows the percentage of living cells after treatment with LPEI– 10kDa normalized on total cell count of untreated cells per well.

This experiment showed that the cytotoxicity of LPEI – 10kDA is dosage dependent. A reduction to less than 50 % living cells could be observed starting with a LPEI concentration of 500 ng/well. Polyplex treatment with both N/P 6 and N/P 9 resulted in no significant reduction of living cells per well compared to untreated cells.

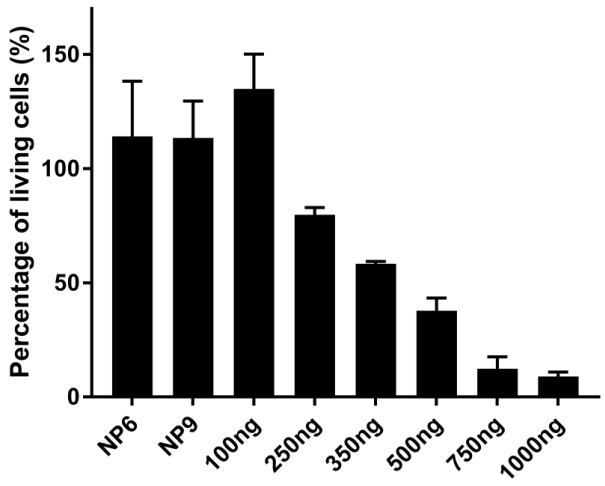


Figure 17: *Percentage of living cells after treatment with LPEI – 10kDa normalized to the number of untreated cells.*

6.5.Transient transfection assay optimization based on A549.wt cells

The testing was carried out on A549.wt cells (CCL-185™, ATCC®, LGC Standards GmbH from Germany). Experiments were conducted using different transfection media, polyplex synthesis buffers, N/P ratios and cell treatment durations (***Table 6***). Wherein the conditions varied with respect to transfection media, buffer, N/P ratio and incubation time:

Transfection medium	1. Basal (=pure) medium (RPMI1640) 2. Complete medium (RPMI1640 + 10 % FCS + L-gln + antibiotics) 3. Opti-MEM®
Polyplex synthesis buffer	1. HBG 2. HBS
N/P ratio	1. N/P 6 2. N/P 9
Cell treatment time	1. 4 hours 2. 24 hours

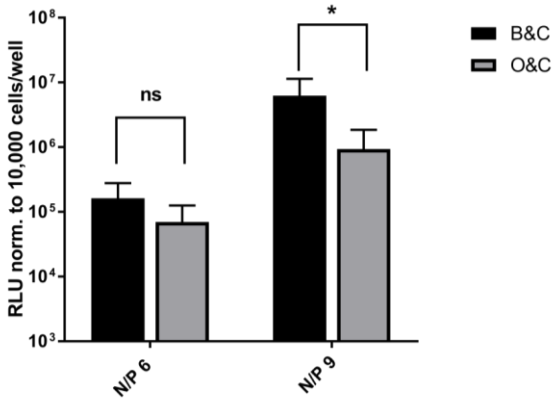
Table 6: List of substances and conditions for section 6.5

1x 10^4 cells per well were seeded into a 96 – well plate (F-bottom, transparent). pCMV Gluc was used as a plasmid DNA component for polyplex generation.
Samples were tested in triplicates and all experiments were performed twice. Experiment protocol is described in section 5.9.1.

The measurement was carried out using flow cytometry for toxicity and plate reader (using the house-made coelenterazine substrate described in section 5.4.). The evaluation was done with FlowJo®, Excel® 2016 and GraphPad®. Statistical evaluation was conducted using U-test (Mann-Whitney). Highest transfection efficiency was obtained for polyplexes with N/P ratio 9 in HBG and B&C as a transfection medium (4 hours incubation time). Opti-MEM as a buffer resulted in reduced transfection efficiency (statistically significant for polyplexes with N/P 9 in HBG and B&C as a transfection medium). Further, polyplexes with N/P ratio 9 prepared in HBG show a higher transfection efficiency than particles prepared

at N/P ratio 6. Polyplexes prepared in HBS show reduced transfection efficiency on A549.wt when compared to polyplexes prepared in HBG.

A



B

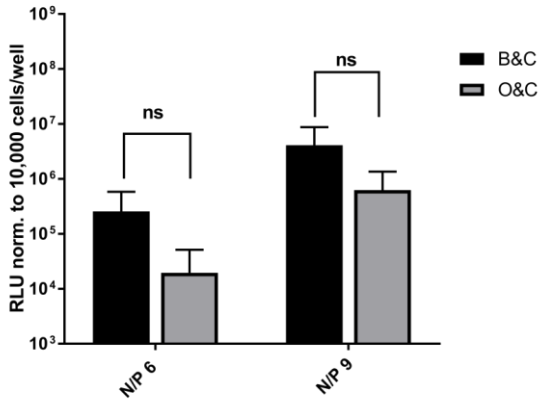


Figure 18a & 18b *Figures show results of testing the transfection efficiency on A549.wt cells in different media with polyplexes made in HBG (**A**) or HBS buffer (**B**) with an incubation time of 4 hours. Data is shown as mean values with standard deviation (n=3, 2 experiments). Statistical evaluation was done using U test (Mann-Whitney). Abbreviations for statistics: B&C: basal and complete medium; O&C: Opti-MEM and complete medium; ns: not statistically significant; * : statistically significant (P < 0.05).*

Based on the testing those findings further transfection studies were conducted using the conditions described in ***Table 7***.

Transfection medium	basal (=pure) medium
Polyplex synthesis buffer	HBG
N/P ratio	N/P 9
Cell treatment time	4 hours

Table 7

6.6. EGFR targeting assays

Targeting assays were performed with A549.wt, CT26.wt and LS174T.wt using conditions described in section 5.9.1. with LPEI (10 kDa), LPEI 10- PEG2- Cys, LPEI 10- PEG2- GE11 and LPEI (10 kDa)- PEG (2 kDa) -- CLARLLT based polyplexes. LPEI (10 kDa) was used as control for positive transfection. LPEI (10 kDa)- PEG (2 kDa)- Cys was used to show detargeting effect due to PEGylation.

6.6.1. Testing of transfection efficiency of LPEI-PEG-CLARLLT on A549.wt

Transfection efficiency was tested with different concentrations of complexed pDNA (50 ng, 100 ng, 200 ng and 400 ng). The experiment described in ***Figure 19*** clearly shows a detargeting effect (based on reduction of RLU [relative light units]) of LPEI (10 kDa)- PEG (2 kDa)- Cys when compared to non- PEGylated LPEI (10 kDa). Using CLARLLT as a targeting component (in LPEI (10 kDa)- PEG (2 kDa) – CLARLLT) results in an increased transfection efficiency when compared to LPEI (10 kDa)- PEG (2 kDa)- Cys. Overall A549.wt cells it is shown with this experiment that A549.wt cells are in general easy to transfect (also with LPEI (10 kDa)- PEG (2 kDa)- Cys). Therefore, significant differences in the transfection efficiency of EGFR targeted and non- targeted LPEI based polyplexes cannot be detected.

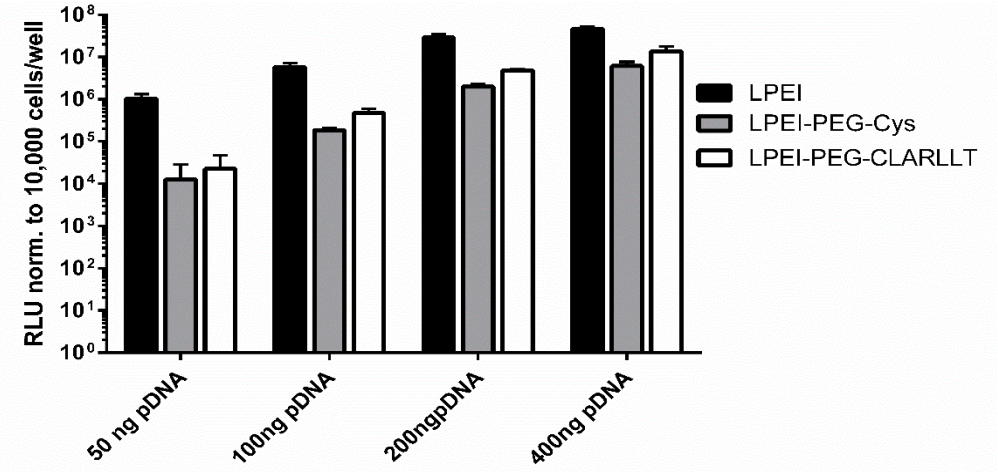


Figure 19 *EGFR mediated transfection study on A549.wt. (n=3,1 experiments) Data are shown as mean values (with standard deviation).*

6.6.2. Testing of transfection efficiency of LPEI-PEG-CLARLLT on CT26.wt

Polyplexes consisting of pDNA (at a concentration of 200 ng/well) and LPEI (10 kDa), LPEI (10 kDa)- PEG (2 kDa) – Cys, LPEI (10 kDa)- PEG (2 kDa) – CLARLLT and LPEI (10 kDa)- PEG (2 kDa) – GE11 in HBG were tested on CT26.wt(shown in ***Figure 20***). An increased transfection efficiency of LPEI (10 kDa)- PEG (2 kDa) – CLARLLT compared to LPEI (10 kDa)- PEG (2 kDa) – GE11 could be detected.

In a further experiment (depicted in ***Figure 21***) components were tested in two different polyplex generation media (HBG, HBS). In both experiments cells were treated for 4 h. Gluc luminescence measurements (plate reader) and toxicity (flow cytometer) were conducted 24 h after adding components to cells. Also, here an increased transfection efficiency of LPEI (10 kDa)- PEG (2 kDa) – CLARLLT based polyplexes prepared in HBG when compared to LPEI (10 kDa)- PEG (2 kDa) – GE11 could be detected. Despite that finding polyplexes of both conjugate types show the direct opposite when prepared in HBS.

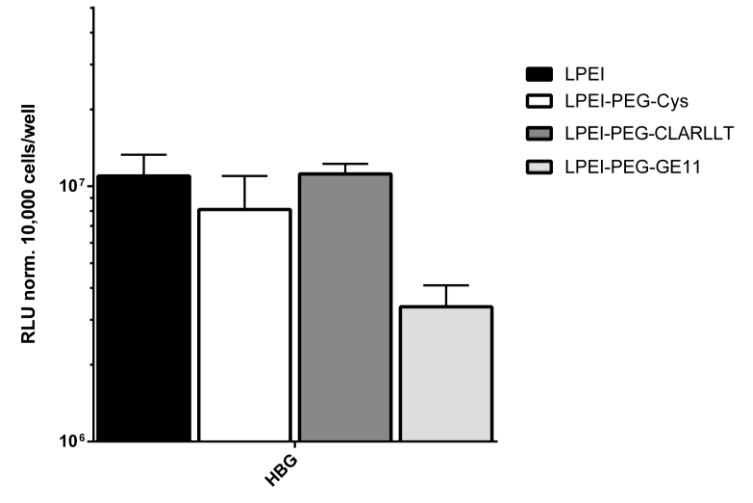


Figure 20*: EGFR targeted transfection experiment on CT26.wt. (4 hours incubation time, 200ng pDNA per well) Data is shown as mean values (with standard deviation) (n=3, 2 experiments).*

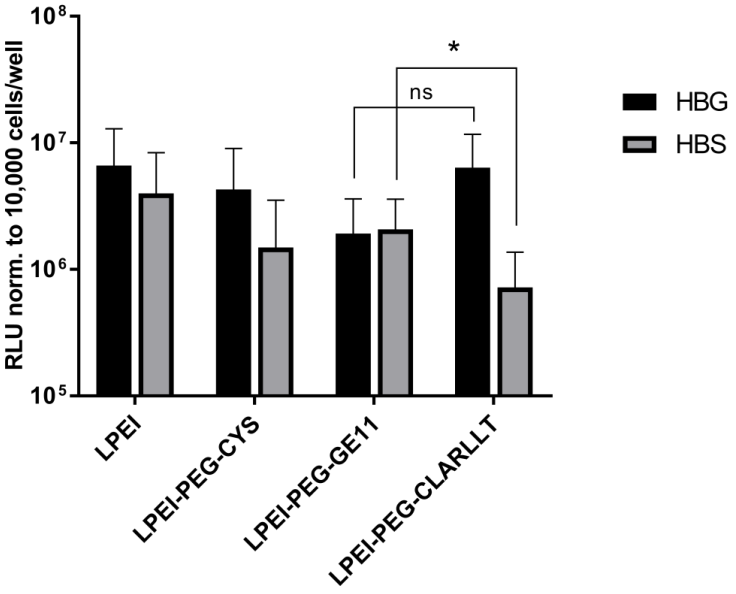


Figure 21: *EGFR targeted transfection study on CT26.wt to show difference of transfection efficiency of polyplexes prepared in HBG and HBS. Data is shown as mean values (with standard deviation) (n=3, 2 experiments). Statistical evaluation was done using U test (Mann-Whitney). Abbreviations for statistics: ns: not statistically significant; * : statistically significant (P < 0.05).*

6.6.3. Testing of transfection efficiency of LPEI-PEG-CLARLLT on LS174T.wt

Compared to all other cell lines used in this diploma thesis LS174T.wt cells highly tend to create cell aggregates and are therefore problematic for checking cellular toxicity of components by flow cytometry (both higher volume of TrypLE® express [40 µl/well; normally 20 µl] and a longer incubation time [8 minutes; normally 5 minutes]. Furthermore, cell suspensions were characterized by high amount of debris (shown in ***Figure 22***)

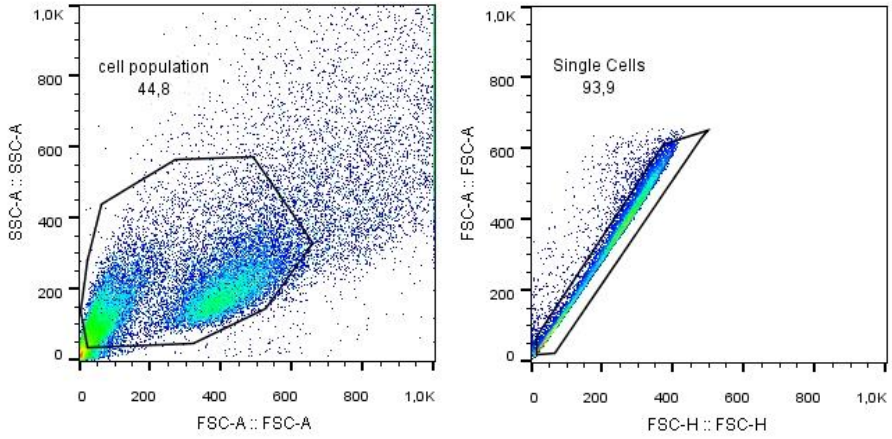


Figure 22: *Representative example for gating strategy of LS174T.wt cells showing the two major problems (high amount of aggregates and debris) when using LS174T.wt for flow cytometry measurements.*

Figure 23 depicts an EGFR targeted transfection study on LS174T.wt cells with different components (LPEI (10 kDa), LPEI (10 kDa)- PEG (2 kDa) – Cys, LPEI (10 kDa)- PEG (2 kDa) – CLARLLT and LPEI (10 kDa)- PEG (2 kDa) – GE11). Here, it is shown that on LS174T.wt GE11 based conjugate works significantly better for EGFR mediated transfection than CLARLLT both at pDNA treatment concentration of 100 ng and 200 ng. But however, both GE11 and CLARLLT based tri- conjugate show a significantly increased transfection efficiency when compared to LPEI (10 kDa)- PEG (2 kDa) – Cys.

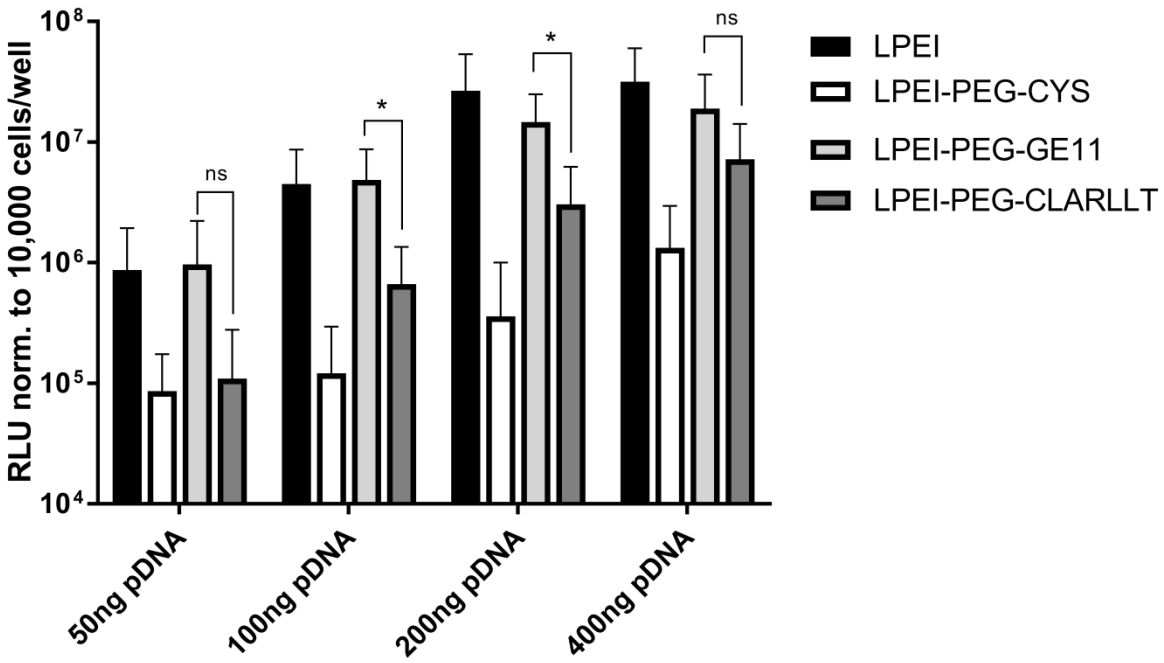


Figure 23: *EGFR targeted transfection experiment on LS174T.wt with different components. Data is shown as mean values with standard deviation (n=3, 2 experiments). Statistical evaluation was done using U test (Mann-Whitney). Abbreviations for statistics: ns: not statistically significant; * : statistically significant (P < 0.05).*

6.7. Nunc chamber optimization on A459.wt cells – establishing a cell fixation protocol

Establishing protocol for cell fixation and antibody staining was done with A549.wt cells. 2x 10^4 cells per chamber were seeded per chamber in a Nunc® chamber slide. Cells were fixed for 30 minutes in 4 % formalin. EGFR antibody staining was conducted with 0.2 µl of AlexaFluor® 488 anti-human EGFR antibody. Co- staining of nucleus was conducted with DAPI (1 µg/ml). Detailed information about the protocol are given in section 5.11. Confocal studies were conducted with an AC3APO 20x 0.06 IMM objective on a Leica TCS SPE confocal microscope. A representative picture is shown in ***Figure 24***.

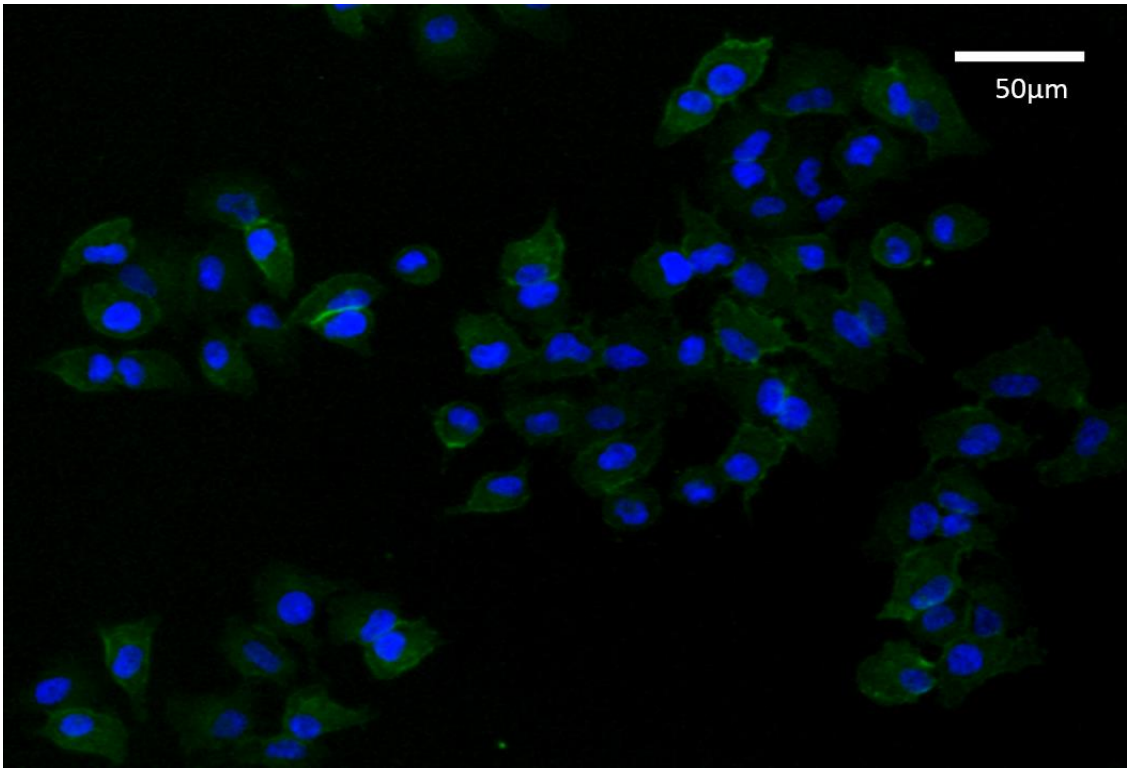


Figure 24: *A549.wt cells fixed with 4% Formalin for 30 minutes and stained with 0.2µl EGFR antibody (AF488, green) and DAPI (blue) (20x magnification).*

6.8. Testing of cell binding /internalization of LPEI 10 – PEG – CLARLLT– uptake evaluation via Confocal Microscopy

Based on binding and uptake studies on the flow cytometer performed by Mag. Alexander Taschauer, cell binding and internalization of LPEI – based polyplexes (LPEI 10kDa/LPEI 10-PEG2- Cys/ LPEI-PEG2- CLARLLT; generated at pDNA concentration 20 µg/ml in HBG) on A549.wt cells were analysed using confocal microscopy.

For visualization of polyplexes p DNA (pCMV - Gluc) labeled with Cy5 was used. $2x10^4$ cells were seeded 24 h prior to treatment. Cells were treated with polyplexed pDNA at an amount of 400 ng per well. Cells were treated for 4hours with polyplex - samples. Afterwards, cell fixation (4% Formalin for 30 minutes) and the counter staining (DAPI) were conducted.

The working process is listed in detail in 5.11.

Figure 25 shows a high binding/uptake of LPEI based polyplexes. Using detargeted PEGylated LPEI (LPEI-PEG-Cys) resulted in clearly visible reduction of cell binding /uptake (***Figure 26***). High cell binding/uptake when using LPEI-PEG-CLARLLT is shown in ***Figure 27***. LPEI–PEG-CLARLLT based polyplexes showed a significantly increased cell binding and uptake, compared to LPEI-PEG-Cys based polyplexes.

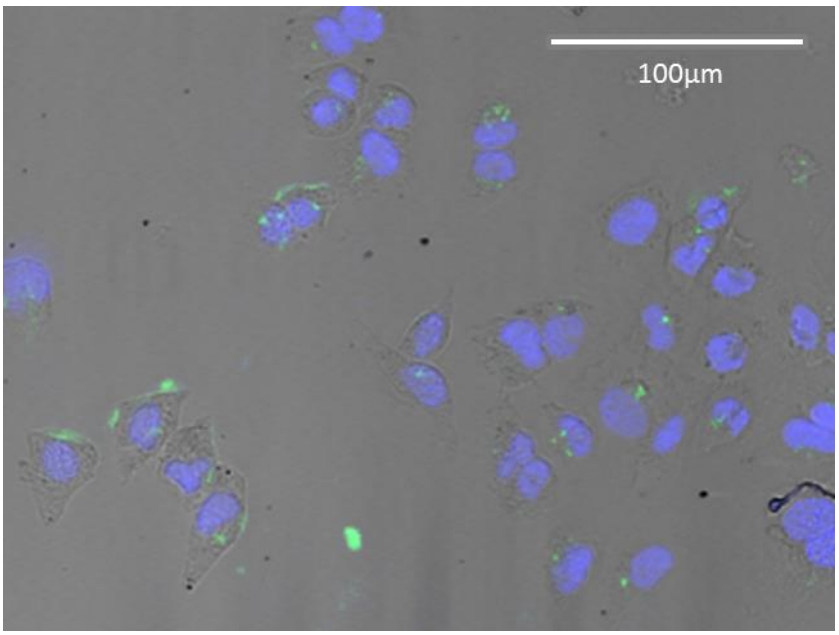


Figure 25: *Representative picture of* ***LPEI 10kDa*** *uptake in A549.wt cells stained with DAPI (20x oil immersion objective). Cell nuclei were stained with DAPI- staining (blue) and the green staining resulted from extracellular human Anti EGFR (Alexa Fluor® 488). The overlay was a DIC (Differential Interference Contrast).*

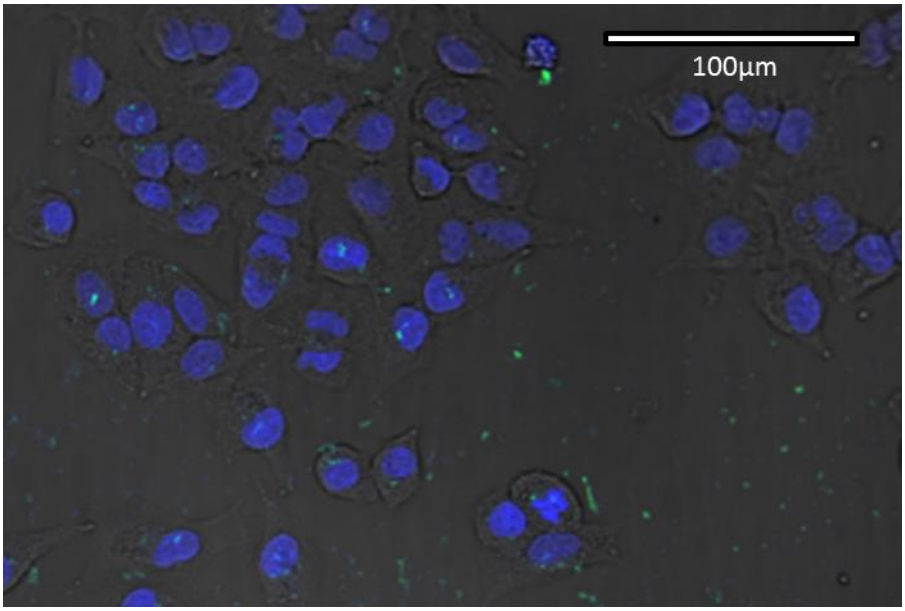
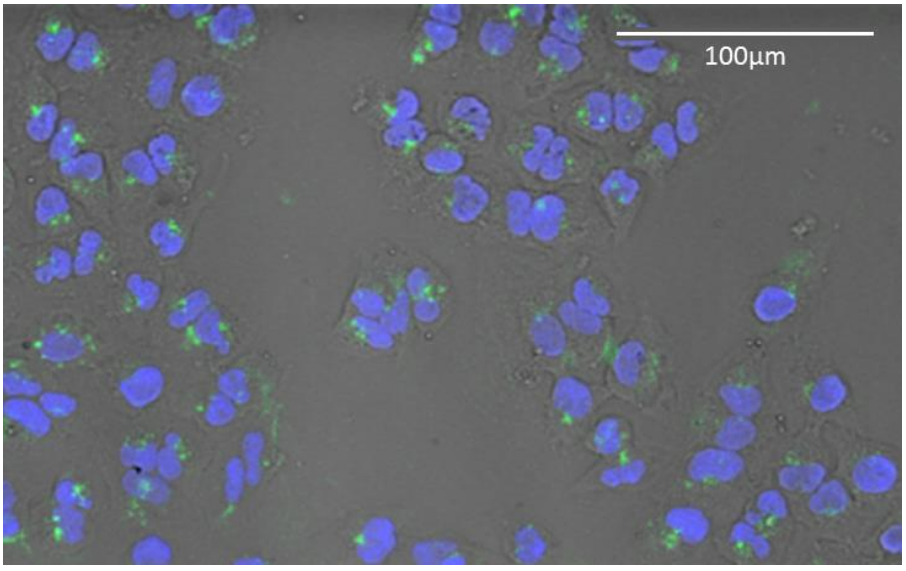


Figure 26: *Representative picture of* ***LPEI 10 – PEG2 – Cys*** *uptake in A549.wt cells stained with DAPI (20x oil immersion objective). Blue staining of the cell nuclei resulted from DAPI- staining and the green staining resulted from extracellular human Anti EGFR (Alexa Fluor® 488). The overlay was a DIC (Differential Interference Contrast).*



Figures 27: *Representative picture of visualization of* ***LPEI 10 – PEG2 – CLARLLT (heptapeptide)*** *uptake in A549.wt cells stained with DAPI (20x oil immersion objective). High cell uptake of the tri-conjugate with CLARLLT could be shown. Blue staining of the cell nuclei resulted from DAPI- staining and the green staining resulted from extracellular human Anti EGFR (Alexa Fluor® 488). The overlay was a DIC (Differential Interference Contrast).*

7. Discussion

EGFR (epidermal growth factor receptor) has become one of the most important targets in anti – cancer treatment in recent years (Song et al. 2009). Several agents of various substance classes (such as Cetuximab (Erbitux®)) have already been established as EGFR – targeting drugs in the clinical field (Song et al. 2009).

As mentioned in the introduction there are still lots of barriers and side effects in cancer treatment, particularly when it comes to conventional chemotherapy (Kang et al. 2015; Borst et al. 2000; Gottesman et al.2002). In recent time, especially several achievements in the field of gene therapy have been made. Glybera® and Strimvelis® are two examples for remarkable clinical progress (Keeler et al. 2017). For gene delivery, two different types of transfection vectors exist, namely viral and non – viral transfection vectors (Ginn et al. 2013; Nguyen and Szoka 2012). Due to the risk of unwanted reactions, such as mutations, the importance for non-viral gene delivery techniques increases (Ginn et al. 2013; Nguyen and Szoka 2012).

The overall aim of this thesis was the biological evaluation of EGFR mediated non – viral gene delivery on both human and murine cancer cell lines
We chose LPEI as complexing agent for pDNA because it has been shown that LPEI is characterized by higher transfection efficiency than BPEI when using pDNA as nucleic acid component of polyplexes (Rödl et al. 2013).

LPEI with a molecular weight of 10kDa was chosen as complexing component, since it is known that high – molecular weight LPEI (over 25kDa) shows a higher cytotoxicity than low – molecular LPEI (Breunig et al. 2007). For all tested polyplexes in this thesis we used pCMV-Gluc as nucleic acid component which has been tested for both sample concentration and quality by UV/Vis spectrophotometry and diagnostic restriction digestion. Furthermore, another important aspect that influences efficiency of both delivery and the toxicity of LPEI had to be considered, , namely the N/P – ratio (Zhao et al. 2009). Because of this we initially focused on testing toxicity of LPEI and on evaluating transfection efficiency of LPEI based polyplexes with N/P 6 and N/P 9.

With increasing concentration of pure LPEI 10 kDa per well cell viability dramatically decreased. A LPEI concentration of 500 ng/well resulted in a reduction of living cells to less than 50 %. But however, cell treatment with polyplexes did not result in significant reduction of living cells per well. Furthermore, there was no significant difference between N/P ratio 6 and N/P ratio 9 in terms of cytotoxicity. Therefore, both N/P ratios were used for further evaluation of optimal cell transfection conditions.

Optimization of transient transfection assay protocol was performed on A549.wt cells (human lung adenocarcinoma). Tests were carried out under different conditions. Different transfection media (basal or complete medium or Opti - MEM®), incubation times for cell treatment (4h or 24h) and N/P ratio (6 or 9) were tested. Best results regarding transfection efficiency were achieved with polyplexes prepared at N/P 9 and generated in HBG for cell treatment in basal medium with 4 hours incubation time. Using transfection media which contain fetal bovine serum (Complete medium and Opti-MEM®) results in reduced transfection efficiency most probably due to interaction of positively charged polyplexes with different serum components. Due to higher amount of free LPEI at N/P 9 higher transfection rate could be achieved when compared to N/P 6. Therefore, for further experiments cells were treated with polyplexes prepared at N/P 9 in basal medium.

Transient transfection of cells with LPEI based polyplexes as characterized by non- specific electrostatic interactions of positively charged polyplexes with the cell membrane (Alexis et al. 2008; Kichler 2004). Different chemical modifications can be used for site-directed transfection. A successful example for chemical modification is functionalization with Polyethylene Glycol (PEG) (Alexis et al. 2008; Kichler 2004). This reduces non- specific interaction with the cellular surface and increases circulation time after iv administration into mice.

A further development for EGF – receptor – mediated gene delivery is the conjugation of a PEGylated LPEI with an EGFR – targeting peptide, such as GE11 and LARLLT. All peptides were conjugated to LPEI-PEG via a disulfide bridge. Therefore, we used peptides functionalized with a N-terminal cysteine. Using such conjugates an EGFR mediated transfection can be achieved (Schäfer et al. 2011).

However, since our work focussed on the biological evaluation of EGFR targeted conjugates on different cancer cell lines, we tested LPEI 10- PEG2- CLARLLT (heptapeptide) on both human and murine EGFR expressing cells. LPEI10-PEG2-GE11 was used as control conjugate. Important, both LARLLT and GE11 are known not to activate EGFR and therefore don´t show mitogenic activity (Normanno et al. 2006; Song et al. 2009).

Since LARLLT sequence has already been tested on human cell lines by Song et al. (2009), we used it as targeting component of LPEI based transient transfection. We tested it on both human and murine cancer cell lines. Conjugates were tested on A549.wt (human lung adenocarcinoma), LS174T.wt (human colon carcinoma) and CT26.wt cells (murine colon carcinoma).

In all cell lines LPEI-PEG-CLARLLT and LPEI-PEG-GE11 based polyplexes showed higher transfection efficiency when compared to non- targeted LPEI-PEG-Cys (Cysteine) based polyplexes. This shows that using EGFR mediated cell transfection can be achieved with both peptides as targeting moiety of LPEI based conjugates. On CT26.wt cells LPEI-PEG-CLARLLT shows higher transfection efficiency than LPEI-PEG-GE11. In case of LS174T.wt cells LPEI-PEG-GE11 based polyplexes are more efficient than LPEI-PEG-CLARLLT. This might be because of slight differences in both EGFR expression levels and in the structure of human and murine EGFR.

Since *Gaussia luciferase* gets secreted, evaluation of luciferase expression can be done with supernatant of cells. Therefore, cells were detached after treated and analyzed by flow cytometry in order to evaluate cellular toxicity of all components. Here, a dose-dependent change in toxicity was observed. Toxicity increased with concentration of polyplexes per well.

Lastly, we could establish a protocol for cell fixation, EGFR antibody staining and DAPI staining in Nunc chamber slides on A549 cells Best results were achieved with Formalin 4% as a fixating agent at an incubation time of 30 minutes. Direct labeling with 0.2 µl of directly labeled EGFR antibody (labeled with AlexaFluor® 488) followed by DAPI treatment at a concentration of 1 µg/ml gave optimal results proved by CLSM.

For binding- and uptake studies of polyplexes Cy5 labeled pCMV-Gluc was used as nucleic acid component of polyplexes. LPEI, LPEI-PEG-Cys and LPEI-PEG-CLARLLT based polyplexes were tested on CT26.wt cells. After 4 hours cells were fixed using the protocol described above. LPEI based polyplexes showed a high cell binding and cell uptake. Compared to LPEI-PEG-Cys (detargeted PEGylated LPEI with a clearly visible reduction of cell binding and uptake), LPEI-PEG-CLARLLT based polyplexes show significantly increased cell binding and uptake.

In summary, we have demonstrated that LPEI – 10 – PEG – CLARLLT conjugate a higher, transfection efficiency then LPEI – 10 – PEG – GE11 on CT26.wt cancer cells.
Furthermore, our experiments have shown that LARLLT peptide sequence does not only work for human, but also for murine cancer cell lines.

8. Appendix

8.1.Abbreviations

Table 9

Abbreviations	full form
(D)PBS	(Dulbecco's) phosphate-buffered saline
(p) CMV – Gluc	(plasmid) Cytomegalovirus Gaussia Luciferase
µg	mikrogram
AB, Ab, ab	antibody
ACC	acceleration
AKH	Allgemeines Krankenhaus
AT	Alexander Taschauer
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pairs
BPEI	branched Polyethylenimine
BRCA1	BReast CAncer Gene 1
BSA	bovine serum albumin
Cat. No.	catalog number
CMC	critical micelle concentration
conc.	concentration
CT	centrifugation tube
ctrl	control
CTZ	Coelenterazine
Cy5	Cyanine 5 dye
Cys	Cysteine
DAPI	4',6-Diamidin-2-phenylindol
DCC	deceleration
ddH_2O	double-distilled water
DMEM – F12	Dulbecco's Modified Eagle's Medium – F12
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOTAP	1, 2- Dioleoyl- 3- trimethylammonium- propane
E. coli	Escherichia coli
e.g.	for example
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
etc.	etcetera
f – bottom	flat – bottom
FBS	fetal bovine serum
FD	fast digest
FSC	Forward Scatter
FSC-A	Forward Scatter - area
FSC-H	Forward Scatter - height
g	gram
HBG buffer	HEPES buffered glucose buffer
HBS buffer	HEPES buffered saline buffer
HP	heptapeptide (CLARLLT)

i.v.	intravenous injection
IgG	immunoglobulin G
kDa	kilo Dalton
KM	Katharina Müller
LAF	laminar air flow
L-gln	L-Glutamin
LB – agar	lysogeny broth- agar
LPEI	linear Polyethylenimin
m	mass
MEME	Minimum Essential *Medium*
mg	milligram
MJ	Martina Joncic
ml	millilitre
mM	milli Molar
mRNA	messenger ribonucleic acid
MW	molecular weight
ng	nanogram
NTA	Nanoparticle tracking analysis
OD	optical density
ODN	oligonucleotide
p16	cyclin dependent kinase inhibitor 2A
p53	tumor suppressor gene with a molecular weight of 53 kDa
PCL	poly- *ε*- caprolactone
pDNA	plasmid deoxyribonucleic acid
PEB	protein extraction buffer
PEG	Polyethylene Glycol
PEI	Polyethylenimine
Pen/Strep	Penicillin/Streptomycin
PLA	polylactide
PLGA	poly- lactic-co-glycolic acid
PnBA	poly- n- butyl acrylate
PPEEA	poly-(2-aminoethyl ethylene phosphate
RFP	red fluorescent protein
RLU	relative light unit
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI – 1640	Roswell Park Memorial Institute - 1640
RT	room temperature
s.c.	subcutaneous injection
SB – buffer	sodium borate buffer
SD	standard deviation
SG	Silvia Gallina
SH	Haider Sami
siRNA	small interfering deoxyribonucleic acid
SSC	Side Scatter
SSC-A	Side Scatter – area
UT	untreated
WEK	purified water
wt	wild type

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