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„Synthesis, analysis, and purification of a targeted DARPin-PAMAM delivery system for siRNA“

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtverletzung bekannt werden, bitte ich um Meldung bei mir.

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List of abbreviations

APS	Ammonium persulfate
BSA	Bovine serum albumin
DARPin	Designed Ankyrin Repeat Protein
DBCO	Dibenzylcyclooctyne
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPC	Dynamic polyconjugate
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EpCAM	Epithelial cell adhesion molecule
FBS / FCS	Fetal bovine serum / Fetal calf serum
GuHcl	Guanidine hydrochloride
h	Hours
Luc	Luciferase
MW	Molecular weight
PAMAM	Polyamidoamine
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEI	Polyethyleneimine
RNAi	RNA interference
RISC	RISC RNA induced silencing complex
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
TBE buffer	Tris/Borate/EDTA buffer
TEMED	Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane

Abstract

Since its discovery, RNA interference (RNAi) has represented a promising pathway to achieve highly efficient downregulation of gene expression by specific interference with the translation of targeted proteins. Small interfering RNA (21-23 base pairs, double-stranded RNA) -based compounds have successfully demonstrated the therapeutic use of the RNAi machinery. However, a variety of additional conjugations and chemical modifications have to be carried out in order to enable targeted siRNA delivery to the diseased tissue, to improve the drug's stability, and to enhance and control the cellular uptake.

For this purpose, the dendrimer PAMAM G4 was conjugated to the DARPin AhaEc1, which binds selectively to EpCAM, to generate a siRNA delivery system that can be specifically recognized by EpCAM expressing tumor cells. To connect the two elements, a DBCO-NHS linker was used.

For analysis of the optimized conjugation reactions, several gel electrophoreses were performed, showing bands of PAMAM-Ec1 conjugates and unbound educts (free protein and linker molecules). Reaction conditions were optimized for effective coupling and adequate yields. Methods for separating reaction products from unreacted components were developed. The best suited method for isolating the conjugates was FPLC purification on an ÄKTA system. Complexes of the conjugates and siRNA were generated by mixing the negatively charged nucleic acid to the dendrimer with its positively charged surface amines. The complexation was not was dependent on charge ratio with the highest loading capacity of 61.7 % reached with N/P 2.

The binding capacity of fluorescent siRNA to EpCAM-positive MDA-MB-468 cells and -negative HeLa cells was tested by flow cytometry and was compared to complexes of the dendrimer and siRNA without the DARPin. The binding of Ec1 containing conjugates was significantly higher in antigen positive tumour cells compared to unconjugated dendrimers, proving selective DARPin mediated cell recognition.

Zusammenfassung

Seit der Entdeckung der RNA-Interferenz (RNAi), stellt diese eine vielversprechende Methode dar, um eine hocheffiziente Downregulierung der Genexpression durch spezifischen Eingriff in die Translation der Zielproteine zu erreichen. Verbindungen, die auf dem Prinzip der Small interfering RNA (doppelsträngige RNA aus 21-23 Basenpaaren) basieren, konnten erfolgreich den therapeutischen Effekt der RNAi-Maschinerie beweisen. Allerdings muss eine Vielzahl von zusätzlichen Konjugationen und chemischen Modifikationen durchgeführt werden, um die gezielte Lieferung siRNA an das erkrankte Gewebe zu ermöglichen bzw., um die Stabilität des Wirkstoffes zu erhöhen und die zelluläre Aufnahme zu steuern.

Zu diesem Zweck wurde das Dendrimer PAMAM G4 an das DARPin AhaEc1, welches selektiv an EpCAM bindet, konjugiert mit dem Ziel, ein siRNA-Trägersystem zu erzeugen, das von EpCAM-exprimierenden Tumorzellen spezifisch erkannt und aufgenommen werden kann. Um die beiden Komponenten zu verbinden, wurde ein DBCO-NHS Linker verwendet. Für die Analyse der optimierten Konjugationsreaktionen wurden mehrere Gelelektrophoresen durchgeführt, welche sowohl Banden von PAMAM-EC1-Konjugaten als auch von ungebundenen Edukten (freiem Protein und Linkermolekülen) zeigten.
Die Reaktionsbedingungen wurden angepasst, um die effektivste Kopplung und die höchst mögliche Ausbeute zu erreichen. Außerdem wurden Verfahren zur Abtrennung der Reaktionsprodukte von weiteren ungebundenen Komponenten getestet. Das am besten geeignete Verfahren zur Isolierung der Konjugate war die FPLC Reinigung unter Verwendung eines ÄKTA Systems. Komplexe aus den Konjugaten und der siRNA wurden durch Mischungen der negativ geladenen Nukleinsäure und des Dendrimers mit positiv geladenen Aminen erzeugt.
Die Komplexbildung war abhängig vom Ladungsverhältnis, wobei die höchste Kapazität von 61,7% durch das Verhältnis N/P 2 erzielt wurde.
Die Bindungskapazität fluoreszierender siRNA zu EpCAM-positiven MDA-MB-468-Zellen und -negativen HeLa-Zellen wurde mit Hilfe der Durchflusszytometrie getestet und mit Komplexen aus siRNA und dem Dendrimer, ohne Verwendung des DARPins verglichen. Die Bindung von Ec1 enthaltenden Konjugaten war im Gegensatz zu PAMAM-siRNA Komplexen deutlich höher in Antigen-positiven Tumorzellen, was die selektive DARPin vermittelte Zell-Erkennung beweist.

1. Introduction

1.1. RNAi and siRNA

In 1998, Andrew Z. Fire and Craig Mello unveiled for the first time the concept of RNA-Interference (RNAi), a mechanism, that is involved in the gene regulation of most eukaryotic cells [1]. First discovered in *Caenorhabditis elegans*, a nematode, RNAi has quickly become a promising field for research, and to develop therapeutics based on that pathway to achieve sequence-specific post-transcriptional gene silencing [1, 2].
So consequently, the use of gene silencing oligonucleotides would lead to simple and effective targeted downregulation, unlike previous more complicated mechanisms [3].

In mammalian cells and organisms, RNAi based application is afforded by small interfering RNA (siRNA).
siRNAs are found endogenously in different organisms on the one hand, or can be synthetically produced to influence a specific messenger RNA (mRNA) on the other one [1].

Characteristic 3'-ends with two overhanging nucleotides enable the detection of siRNA by the enzymatic machinery of the cell. In mammalian cells, Dicer, a RNAse III endonuclease cleaves long double stranded RNA into smaller fragments, producing siRNAs, which are then loaded to the Argonaute protein Ago2, an element of the so called RNA-induced Silencing Complex (RISC), besides other components like the TAR-RNA Binding protein (TRBP) [1, 4, 5].

One strand, the "guide" strand is selected to remain bound to Ago2, while its counterpart, the “passenger” strand, is cleaved [1]. RISC is then guided to specifically bind the complementary mRNAs. The targeting is exact, because it is achieved by Watson-Crick base pairing between the siRNA and target mRNA. Generally the two sides show complementarity to each other. After binding, Ago catalyzes the cleavage of mRNA, which is consequently degraded, and that is basically the way siRNAs can silence gene expression [4, 6, 7].

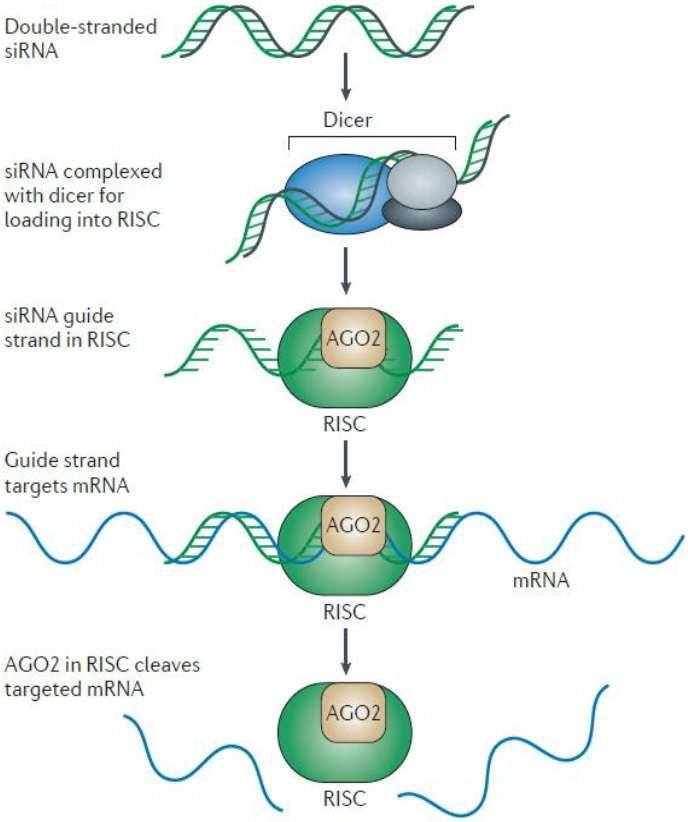


Figure 1: small interfering RNA - mechanism [8]

In 2004 Acuity pharmaceuticals (now OPKO) presented *bevasiranib*, an intravitreal injection of siRNA with the therapeutic aim to target vascular endothelial growth factor (VEGF) mRNA in patients with age-related macular degeneration (AMD). It became the first ever siRNA-based drug compound to reach Phase III clinical trial. However, the trial was stopped in 2009, after the confirmation that it was not sufficiently effective in reducing vision impairment [9]. In particular, difficulties as low stability, an insufficient cellular uptake and doubtful specificity have been reported [10].

Several other companies have also attempted the use of RNAi based therapeutics with the target to overcome previously mentioned obstacles concerning siRNA delivery.
Rxi Pharma, as an example, has aimed at the development of siRNA alternatives, like Self-Delivering RNAi compounds *sd-rxRNA* [9]. Compared to typical siRNAs, sd-rxRNAs have a small double stranded sequence of less than 15 base pairs in addition to a single stranded phosphorothioated tail. Moreover, bases and end groups are modified with various hydrophobic and stabilizing moieties. This combination of RNAi and conventional antisense technologies result in optimized, size-reduced molecules with improved circulation and tissue uptake and penetration. Their chemical modifications enable the uptake by cells without the requirement for delivery vehicles, and protect them against nucleases. [11]

Due to their charge, size and chemical structure oligonucleotides are not capable of bypassing the cellular plasma membrane, and would consequently be quickly degraded by nucleases in blood after application. So, tremendous efforts have been made to develop delivery systems that are able to guide oligonucleotides to their target location in cytosol. [10, 12]

1.2. Cellular uptake

One of the major obstacles remains to deliver siRNA compounds to the targeted diseased tissue. In order to reach specific cellular uptake, fusion proteins, containing a cell-recognizing domain and an element for RNA-interactions, have been developed and recently tested in clinical trials. [10]

Generally, the main advantage of proteins in compounds is their ability to enable much higher specificity to bigger proteins, such as protein kinases, by recognizing and binding to characteristic functional groups. Usually small molecules don't have the opportunity to reach these targets, as the majority of target proteins are able to interact with other proteins, but may not have the required binding pockets for small molecules. Nevertheless, proteins do not have the capacity to pass cell membranes, and have to be attached to other delivery tools for intracellular applications. [13]

Antibodies or AB fragments have been commonly used as delivery vehicles for tumor targeting compounds. Although, there have been concerns associated with their low expression and their tendency to aggregate. In order to bypass these limitations, DARPins were firstly developed. [14]

Designed Ankyrin Repeat Proteins (DARPins) are a recent popular class of binding molecules, which show advantageous biophysical characteristics like high affinity, stability, and high expression yields in simple organisms such as E.Coli. DARPins originate from naturally existing ankyrin proteins and consist of internal repeat units, representing the binding part, and N- and C- terminal capping repeats to supply solubility and stability. These properties make them ideal molecules for targeting tumors.

Indeed, specific DARPins have been developed to bind to the epithelial cell adhesion molecule (EpCAM), a 40 kDa membrane glycoprotein, which is highly expressed in cells of breast, colon and pancreas carcinoma. [15] A fusion protein containing an EpCAM specific DARPin with a shortened protamine sequence has been produced and tested. The conjugate succeeded to deliver the attached siRNA specifically to EpCAM-expressing tumor cells and led to downregulation of Bcl-2, the targeted antapoptotic tumor protein. [16]

1.3 siRNA therapies

At the moment, the siRNA therapeutic, that has reached the most advanced level in clinical trials, is *patisiran (ALN-TTR02),* developed by Alnylam Pharmaceuticals, which is currently in phase III. Patisiran has been developed as a lipid nanoparticle formulation for treatment of transthyretin (TTR)-mediated amyloidosis (ATTR). Its mechanism of action is based on the specific knockdown of both wild type and mutant forms of TTR, whose mutations cause the disease, which can lead to polyneuropathy or cardiomyopathy and consequently heart failure. [3, 17]

In phase II, a significant dose-dependent knockdown of TTR was proven. The reduction after the application of two doses reached 85%, while a maximum knockdown of 96% was observed in patients, who received a single dose every 3 weeks.
In November 2013, the APOLLO Phase III trial of patisiran was initiated for evaluation in ATTR patients with familial amyloidotic polyneuropathy (FAP).

Another anti-TTR-siRNA compound, *revusiran* (*ALN-TTRsc)* is currently developed (phase III) by the same company as a N-acetylgalactosamine (GalNAc) bioconjugate for subcutaneous application for the treatment TTR-amyloid-induced cardiomyopathies. The covalent attachment of GalNAc to siRNA promises a high affinity to the asialo glacoprotein receptor and thus a higher cellular uptake.

In the field of viral diseases, Arrowhead Research Corporation is evolving an anti-HBV-siRNA, *ARC-520* again using GalNAc, with the major difference, that N-acetylgalactosamine is not covalently linked to siRNA. Instead, the company is developing so called "dynamic polyconjugates", a polymer which is covalently linked to the targeting GalNAc,

and complexed to siRNA and is degraded in the acidic environment of the endosome. This new strategy is designed to increase the endosomal escape of siRNAs. [3, 18, 19]

1.3.1. siRNA Delivery systems

Lipid based delivery

To overcome some of the previously mentioned difficulties in siRNA delivery, a lot of research has been done on developing lipid-based delivery systems, as liposomes have been frequently reported as very successful vehicles for drugs and gene agents. [20]
Principally this type of delivery promises a protection of siRNA from nuclease degradation in the blood stream and during internalization, and the improved penetration into cells by crossing their cell membranes, especially in case of systemic application. [9, 21]

Actually, the most broadly explored and used transfection reagents for nucleic acid delivery, are cationic *lipid based nanoparticles (LNPs)* due to their ability to build electrostatically induced complexes with negatively charged backbones of oligonucleotides without any complications. [22]
One common ground of LNPs is the typical construction, consisting of a cationic head group, a hydrophobic segment, and a linker connecting both groups. [23]

The first synthetic lipid used for delivering plasmid DNA into mammalian cells was *DOTMA (N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride).* In addition *DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane)* and *Transfectam* were used later on. These lipid carriers have shown significant advantages concerning the escape of siRNA from the endosomes into cytoplasm.

The higher release results from the tie between cationic lipids with cellular anionic lipids and the associated neutralisation. [9] However, despite their high delivering potential, cationic LNPs have demonstrated toxicity and unintended uptake by the liver after systemic administration, caused by their cationic surface groups.
As a possible replacement neutral *lipid based liposomes* have been developed, and have also shown a great capability as transfection reagents, but without noticeable toxicity. Still, in the field of cancer, these nanoparticles have not been ideal. Due to their size of 80 to 150 nm, their capacity to reach and penetrate tumor tissue is restricted. [22]

Recently, *small lipid nanoparticles (SLNPs)* have been produced as advanced carriers for siRNA. SLNPs consist of a cationic cholesterol derivate, mono-arginine-cholesterol (Ma-Chol) as the main lipid element, in addition to a neutral assisting lipid, dioleoyl phosphatidylethanolamine (DOPE), and a low amount of PEGylated phospholipids combined with the required siRNA.
The resulting nanoparticles are neutrally charged, clearly less toxic than the typical cationic cholesterol based carriers, and have the size of less than 50 nm. So they have also been used in cancer therapy, either by downregulating target genes, or in systemic injections, targeting tumor tissue. [22]

Dynamic Polyconjugates
The most recently developed siRNA delivery platform is called Dynamic Polyconjugate (DPC). These synthetic polymer-based formulations were generated, based on the model of viruses, for their ability to find target cells and transfer their own nucleic acids to accurate compartments of the cells. [24, 25]

DPCs are small nanoparticles measuring 5 - 20 nm. They consist of an amphipathic polymer, which is reversibly attached to protecting agents, for example PEG, in addition to targeting ligands and the siRNA. The choice between different polymers is contingent on their ability to lyse membranes, which is dependent on the ratio of positive charges of amines to hydrophobic domains. [26]
Usually the amines are masked with maleic anhydride derivatives called CDMs, in order to create acid-labile malemate groups with minimal negative charge. [27] In the endosomal acidic environment, these bonds are cleaved and the amines of the polymer are unmasked, following the activation of its lytic capacity.

Summing up, the main objectives of masking the amines are reducing toxicity by regulating the exact time of membrane lysis according to the activation of the polymer and avoiding interactions with non-targeting cells and blood components. [19, 26] The final aim of DPCs is reached when the siRNA, which is either linked to the polymer by a disulfide bond or complexed, is released as a consequence of membrane degradation, to the cytoplasm, where they can lead to the knockdown of target gene expression using the cell's RNAi machinery.

The first endosomolytic polymer that was used for DPC-development was composed of poly butyl and amino vinyl ethers (PBAVE). [28] It was attached to N-acetyl-galactosamine (NAG) to target liver hepatocytes.

Due to the high affinity of the NAG-ligand for the asialoglycoprotein receptor, which is exclusively expressed in hepatocytes, a specific cellular uptake was possible.[27, 28] Later, more sophisticated polymers were produced, especially in relation to a homogenous size, which was not possible in case of PBAVE due to uncontrolled polymerization.

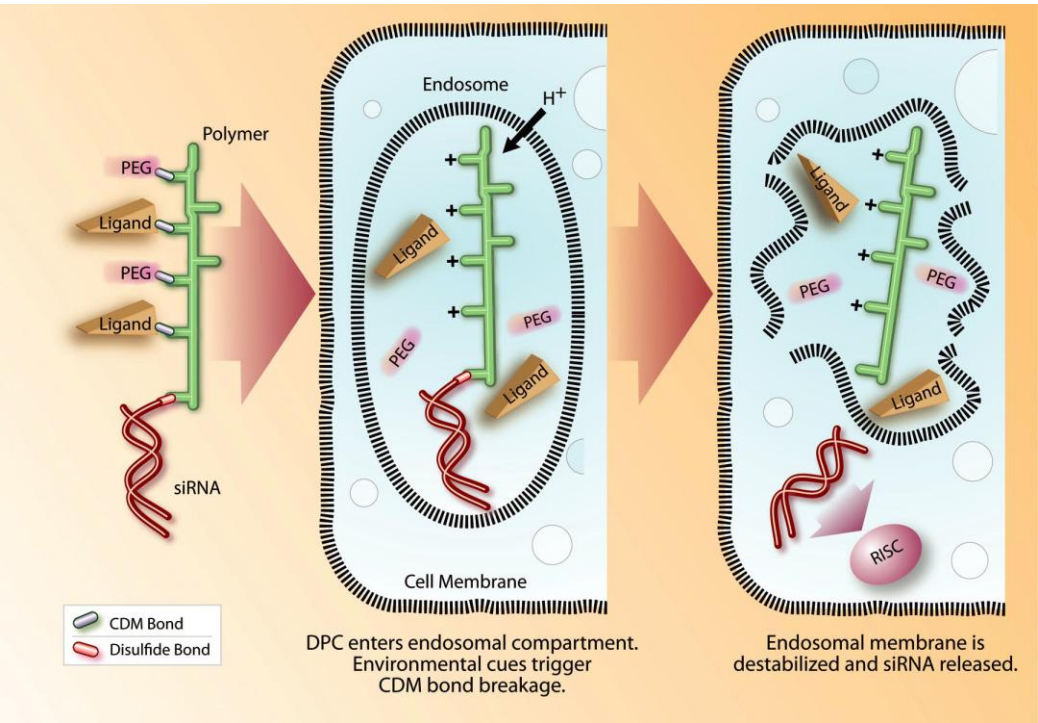


Figure 2: Dynamic Polyconjugates [27]

Despite the focus on developing DPCs for hepatic diseases like hepatitis B infections, new methods have been recently evolved to attach other targeting ligands to the polymers, like peptides, glycans, lectins, small molecule drugs and antibodies. This advancement would enable targeting other types of cells, such as tumors, whereas the small size of DPCs is of great benefit for that purpose. [24, 26] Arrowhead Research Corporation, the company, that first introduced DPCs, aims continuous improvement of the siRNA delivery systems to develop, inter alia, identifying ligands for efficient targeting of tumor tissues. [26]

Dendrimers

Dendrimers represent a popular and quite recent class of drug and siRNA delivery systems. Originally deriving from the Greek word "dendron", meaning tree, Dendrimers are defined as synthetic, nanoscopic polymers. [29] They are usually characterized by their nanometric size (1-100 nm), their extremely symmetric and well defined three-dimensional structure with a high number of branched layers and a high density of surface charges. [30, 31]

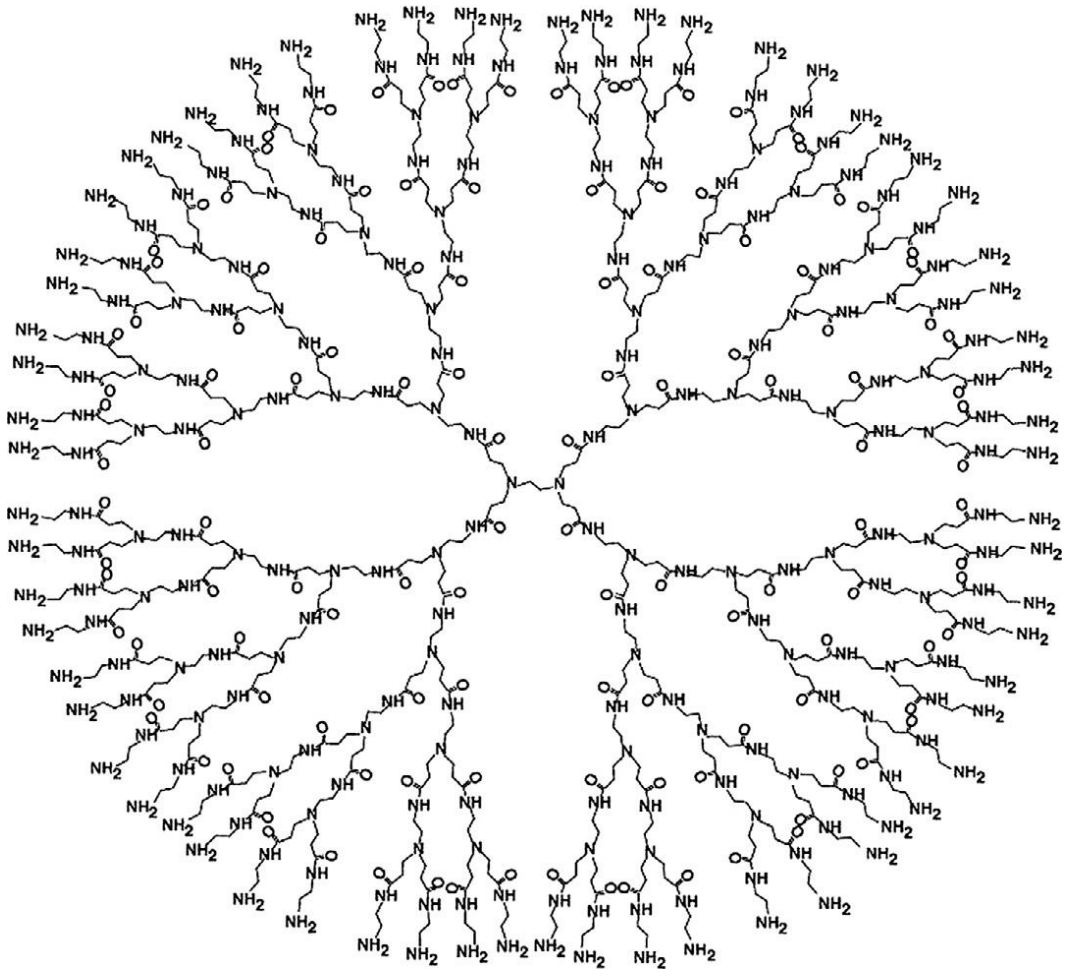


Figure 3: PAMAM G4 [31]

A typical dendrimer contains three elements:

- *The core*, which includes one or several atoms, or mostly a molecule, and is located in the heart of the dendrimer
- *The branching units*: are covalently connected to the core compound. The repetition of branching cycles leads to sequences of concentric layers. The number of these cycles is called generation.
- *The terminal functional groups* at the exterior part of the dendritic structure

Consequently, with each subsequent generation, the size of the dendrimer increases linearly, while the number of surface functional groups rises exponentially. [30]

Generally there are three ways for drugs to interact with dendrimers:

- *Physical encapsulations*: Due to empty cavities in the structure, especially hydrophobic molecules can be enclosed in the interior of the dendrimer. These hollow spaces are primarily available in lower generations
- *Electrostatic interactions* between charged surface groups and hydrophilic drugs and
- *Covalent conjugations* to surface functional groups building structures called pro-drugs. Once internalized into the target cells, the conjugate has to be cleaved to release and activate the drug [29, 30]

Generally dendrimers can be synthesized by two major pathways. In the divergent method, the synthesis starts from the core and expands outwards by a step-by-step addition of forming sections. In the convergent method, the synthesis starts with the exterior functional groups, implying a predetermination of the final generation number, and advances towards the core. [32]

The two most commonly used dendrimers for delivering RNAi therapeutics are Polyamidoamine (PAMAM) and Polyethyleneimine (PEI).
PAMAM is based on an ethylenediamine core and surface amino groups, representing a high density of terminal positive charges. These cationic groups show a great ability to build strong, but reversible complexes with oligonucleotides, such as siRNAs, based on electrostatic interactions with negatively charged surface groups. Comparing different generations of PAMAM, for example G3 and G4, an increased binding energy between the dendrimer and siRNA was observed, when the higher generation was used. [31]

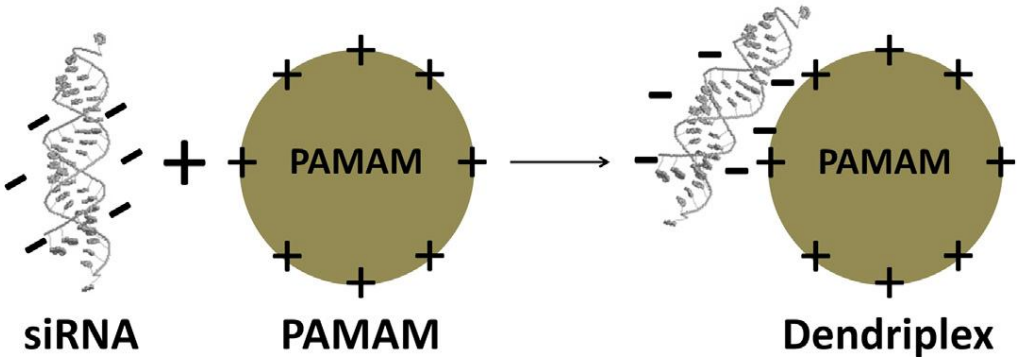


Figure 4: Electrostatic interaction between PAMAM and siRNA [31]

The so called "dendriplexes" are capable of crossing cell membranes, particularly due to their small size. In addition, PAMAM has the ability of protecting siRNAs from degradation by RNAses during delivery. Several studies proved that complexes built at the N/P ratio (ratio of nitrogens of PAMAM to phosphates of siRNA) were not enzymatically degraded. After cellular entry, oligonucleotides are released from the endosomes by the "proton-sponge-effect", which is based on the osmotic swelling of endosomes, induced by PAMAM, in order to finally exert their effect and knock down target genes. [31]

PEI has also been successfully used as a delivery vector for nucleic acids, again by electrostatic interaction induced by multiple protonable amino surface groups. On the basis of its high pH buffering ability, it has shown a high endosomal escape rate. [33]

Despite the wide use of PAMAM in the area of drug delivery, there has been considerable evidence of in vitro cytotoxicity related to the dendrimer. The high number of positive surface charges obviously leads to an interaction with negatively charged domains of biological membranes, resulting in membrane damage by building nano-holes, disintegration or membrane thinning. On the other hand, positive charges are essential for the conjugation with therapeutic agents. Generally, toxicity of dendrimers has been reported to be dependent on generation, dose, and number and type of the terminal groups.

It has been shown, that the toxic effects of PAMAM can be reduced by modifying the surface groups with carbohydrates, acetate or PEG. Eventually, toxicity should be weighed against the benefits of dendrimers in gene transfection. [31, 34]

2. Aim

The primary objective of this work was to generate a high capacity, targeted siRNA delivery system using PAMAM dendrimer and an EpCAM specific DARPin. In diploma thesis, the improvement of existing procedures for conjugating DARPins and PAMAM and the isolation of pure conjugates of PAMAM and the protein (AhaEc1) were planned.

For binding PAMAM to DARPin, a DBCO linker was used, as it shows great ability to easily connect both molecules by active ester mediated attachment to the amino surface groups of PAMAM, and by simple click chemistry for the conjugation with the azido-modified protein. The linker should first be attached to PAMAM, followed to conjugation to a DARPin with an N-terminal azidohomoalanine-modification.
As for the conjugation of the dendrimer and the linker, the conditions for the reactions had to be optimized, as it is known to advance slowly and with a moderate yield. The success of all binding reactions was tested with spectrophotometrical or biochemical methods. For example, SDS-PAGE was previously proven to be suited for verifying protein conjugations.

Also the conjugations of AhaEc1 to the dendrimer had to be improved to create the best possible conditions for the educts to react completely, or at least, separate the obtained products from secondary products. The binding capacity of PAMAM for siRNA by electrostatic interaction between phosphate and amino groups was to be investigated.

The performance of cell culture tests was intended to check the cellular uptake of the produced compounds by antigen-positive and negative cells and consequently be able to estimate the role of all components, especially PAMAM and the DARPin.
So, overall the aim was to develop PAMAM-Ec1-siRNA conjugates as a specific delivery system for receptor mediated cellular uptake.

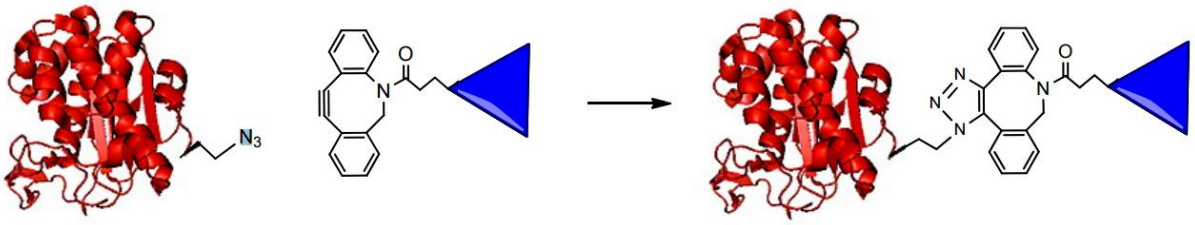


Figure 5: Schematic Conjugation of DBCO-linker to Aha-Ec1 (red) and PAMAM (blue)

3. Materials and instruments

3.1. Chemical methods

Click chemistry: click conjugation of PAMAM dendrimer to DBCO-linker

- PAMAM dendrimer, ethylenediamine core, generation 4.0 solution, 10 wt. % in methanol - Dendritech® Inc. Sigma-Aldrich Steinheim, Germany
- Linker: 10 mM in DMSO: DBCO-NHS-linker (402.4 g/mol) - Sigma-Aldrich Steinheim, Germany
- Sodium tetraborate buffer pH 8.5, 50 mM
- 1 x PBS buffer, 1:10 dilution of: DPBS (10x), Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride - GIBCO®, Life Technologies Corporation

Instruments:

- Rotational-Vacuum-Concentrator RVC 2-18 CD - Christ Gefriertrockungsanlage Alpha 2-4 LSC Freeze dryer
- ND-1000 Nanodrop® Spectrophotometer peqlab - Biotechnologie GmbH
- Thermomixer comfort - Eppendorf
- Slide-A-Lyzer® Mini Dialysis Units, 2.000 MWCO - Thermo Scientific
- Amicon® Ultra 0.5 mL Centrifugal Filters - Merck Millipore, Merck KGaA
- Centrifuge 5810 R - Eppendorf

Binding Aha-Ec1 to PAMAM

- Aha-Ec1 (18344.11 Da) – provided by University of Zurich

Components	**Amount**
Guanidine-HCl	3 M
TRIS	10 mM
Nuclease free water	to 250 ml
pH 8.0 was set and the solution was filtered	

- Guanidine hydrochloride, pH 8.0, 3M

siRNA complexation (including rebuffering)

- ➜ PAMAM-Ec1 conjugates (stock solution in 3M GuHCl)
- ➜ PAMAM G4 (1 mg/10 µl stock solution in water)
- ➜ Anti-Luc-siRNA Duplex, (AS_UCGAAGUACUCAGCGUAAGdTdT - S_CUUACGCUGAGUACUUCGAdTdT) - GlaxoSmithKline Pharma GmbH
- ➜ 1 x PBS buffer, 1:10 dilution of: DPBS (10x), Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride - GIBCO®, Life Technologies Corporation
- ➜ 10 % Polyacrylamide gel in 1x TBE buffer

Components	**Amount for one gel**
40 % acrylamide : acrylamide 193.1 g + bis-acrylamide 6.9 g + ddH_2O 500 ml	1.875 ml
TBE buffer 10x : Tris 890 mM (107 g) + Boric acid 890 mM (55 g) + EDTA (pH 8.0) 25 mM (5.8 g) ddH_2O to 1000 ml	0.75 ml
ddH_2O	4.70 ml
TEMED	10 µl
APS 10 % (w/v)	100 µl

- ➜ DNA Loading Dye 6x

Components	**Amount**
Bromophenol Blue Na-salt	0.03 %
Tris-HCl (pH 7.6)	10 mM
Xylene cyanol	0.03 %
Glycerol	60 %
EDTA	60 mM
ddH_2O to	1000 ml

- ➜ Methylene blue staining solution

Components	**Amount**
Methylene blue	20 mg
TBE buffer 10x	1 ml
ddH_2O	to 100 ml

Instruments:

- ➜ BIO-RAD glass plates and combs
- ➜ BIO-RAD Power PAC 1000 and Mini-PROTEAN® 3 Cell
- ➜ BIO-RAD GS-710 Calibrated Densitometer

3.2. Biochemical methods

Gel electrophoresis SDS-PAGE including sample purification

- ➜ Sephadex-G50 Gel (200 µl for each sample)
- ➜ Nuclease free water
- ➜ 12 % SDS-polyacrylamide Gel
  - **12 % Separating gel**

Components	**Amount for one gel**
Tris HCl: TRIS 90.80 g pH 8.8 +SDS 2.00 g + ddH_2O to 500 ml	1.25 ml
Acrylamide 30 % - Bis-acrylamide 0.8 % (w/v): 2x acrylamide 120 ml + 2x bisacrylamide 3.2 ml + ddH_2O to 400 ml	2 ml
ddH_2O	1.72 ml
APS 10 % (w/v)	3.3 µl
TEMED	25 µl

  - **4 % Stacking gel**

Components	**Amount for one gel**
Tris HCl : TRIS 6.06 g + pH 6.8 SDS 0.4 g + ddH_2O to 100 ml	0.5 ml
Acrylamide 30 % (w/v) / Bis-acrylamide 0.8 % (w/v)	0.268 ml
ddH_2O	1.23 ml
TEMED	2 µl
APS 10 % (w/v)	10 µl

➜ Laemmli Buffer 4x

Components	Amount
TRIS pH 6.8	250 mM
SDS	8 %
Glycerol	40 %
Bromphenol blue	0.02 %
DTT	6.2 mg/ml

➜ PageRuler™ Prestained Protein Ladder standard, 10 to 180 kDa - Thermo Fisher Scientific, includes Dye-stained proteins with specific MW of:
10 kDa, 15 kDa, 25 kDa, 35 kDa, 40 kDa, 55 kDa, 70 kDa, 100 kDa, 130 kDa, 180 kDa The standard indicates two reference bands: 10 kDa (green) and 70 kDa (orange). The protein are kept in 62.5 mM Tris-H_3PO_4 pH 7.5 (at 25°C), 1 mM EDTA, 2% SDS, 10 mM DTT, 1 mM NaN3 and 33% glycerol.

➜ Anode buffer 10x

Components	Amount
TRIS	30.2 g
SDS	10.0 g
ddH_2O	to 1000 ml
A 1:10 dilution was used (1x buffer)	

➜ Cathode buffer 10x

Components	Amount
TRIS	30.2 g
SDS	10.0 g
Glycine	144.2 g
ddH_2O	to 1000 ml
A 1:10 dilution was used (1x buffer)	

➜ Coomassie staining solution

Components	Amount
Coomassie® Brilliant Blue G 250	0.1%
ddH_2O	50 %
Acetic acid	10 %
Methanol	40 %

➜ Coomassie destaining solution

Components	Amount
Acetic acid	10 %
ddH_2O	50 %
Methanol	40 %

Isolation of PAMAM-Ec1-conjuagtes by ÄKTA

➜ PAMAM-Ec1-conjugates stock solution in 3M GuHCl

➜ Guanidine hydrochloride, pH 8.0, 3M

Instruments:

➜ ÄKTA™ pure - GE Healthcare Life Sciences

➜ Software: UNICORN 6.3

➜ Superdex™ 75 10/300 GL - GE Healthcare Life Sciences

➜ Sample loop 500 µl - GE Healthcare Life Sciences

➜ Syringe 1 ml - B. Braun Austria GmbH

Collection and gel electrophoresis of isolated compounds after ÄKTA purification

➜ Fractions after purification eluted with GuHCl

➜ Sephadex-G50 Gel (200 µl for each sample)

➜ Nuclease free water

➜ 12 % SDS-polyacrylamide Gel

➜ Anode buffer 10x

➜ Cathode buffer 10x

➜ Coomassie staining solution

➜ Coomassie destaining solution

3.3. Cell culture

Luciferase Assay

Cell splitting and seeding

- MDA-MB-468 cells - supplied by ATCC®
- HeLa cells - supplied by ATCC®
- DMEM 1x, Dulbecco's Modified Eagle Medium + GlutaMAX™ 500ml [+] 4.5 g/l glucose, [-] pyruvate - GIBCO®, Life Technologies Corporation + 10% Serum FBS - GIBCO®, Life Technologies Corporation
- TrypLE™ Express, stable Trypsin Replacement Enzyme 100 ml - GIBCO®, Life Technologies Corporation
- Dulbecco's 1x PBS, Phosphate Buffer Saline without calcium chloride and magnesium chloride - GIBCO®, Life Technologies Corporation

Instruments:

- Incubator CO_2 6000 - NAPCO®

Plasmid transfection

- Opti-MEM® I Reduced Serum Medium, w/o Phenol Red - GIBCO®, Life Technologies Corporation
- DMEM 1x, Dulbecco's Modified Eagle Medium + GlutaMAX™ 500ml [+] 4.5 g/l glucose, [-] pyruvate - GIBCO®, Life Technologies Corporation
- Lipofectamine™ 2000, 0.75 ml - 1 mg/ml - Invitrogen™, Life Technologies Corporation
- EGFPLuc - plasmid DNA
- CMV-Gluc - plasmid DNA

Sample transfections

- Opti-MEM® I Reduced Serum Medium, w/o Phenol Red - GIBCO®, Life Technologies Corporation
- Lipofectamine™ 2000, 0.75 ml, 1 mg/ml - Invitrogen™, Life Technologies Corporation
- Lipofectamine™ RNAiMAX, 1.5 ml - Invitrogen™, Life Technologies Corporation
- PAMAM-Ec1-siRNA conjugates - stock solutions in 1x PBS
- Anti-Luc-siRNA double stranded, GlaxoSmithKline Pharma GmbH

Luminescence Readout

- Passive lysis buffer 5x 30 ml - Promega
- Luciferase Assay Reagent 1 vial lyophilized product - Promega
- Luciferase Assay Buffer II 10 ml – LAR II - Promega
- Stop and Glo Substrate 50x 200 µl - Promega
- Stop and Glo buffer 10 ml - Promega

Instruments:

- Infinite M 200 PRO - Tecan Trading AG, Switzerland

Uptake of fluorescent siRNA (FACS)

siRNA complexation

- PAMAM-Ec1 conjugates (stock solution in 3M GuHCl)
- PAMAM G4 (1 mg/10 µl stock solution in water)
- Alexa fluor 568 labeled Luc AS siRNA (AS_ UCGAAGUACUCAGCGUAAGdTdT - S_CUUACGCUGAGUACUUCGAdTdT)
- SS siRNA (S_ CUUACGCUGAGUACUUCGAdTdT)
- 1 x PBS buffer, 1:10 dilution of: DPBS (10x), Dulbecco's Phosphate Buffer Saline without calcium chloride and magnesium chloride - GIBCO®, Life Technologies Corporation

Transfection of cells and fluorescence readout

- PAMAM-Ec1-siRNA conjugates - stock solutions in 1x PBS
- Alexa fluor 568 labeled Luc AS siRNA
- TrypLE™ Express, stable Trypsin Replacement Enzyme 100 ml - GIBCO®, Life Technologies Corporation
- Dulbecco's 1x PBS, Phosphate Buffer Saline without calcium chloride and magnesium chloride - GIBCO®, Life Technologies Corporation
- Dulbecco's 1x PBS, Phosphate buffered saline without Ca and Mg - GIBCO®, Life Technologies Corporation + 1 % Serum FCS - GIBCO®, Life Technologies Corporation

Instruments:

- BD FACSCalibur™ Flow Cytometer

4. Methods

4.1. Click chemistry

4.1.1. Preparation of dendrimer stock

The dendrimer, which was used in these experiments, was PAMAM G4 (generation 4).
The molecular weight of PAMAM G4 is 14215 g/mol and its solution in methanol has a density of 0.813 g/ml.
This generation of the dendrimer has 64 amino surface groups.
To prepare a water stock solution, an aliquot of a methanolic PAMAM G4 solution (10%) was put in the rotational vacuum concentrator until the solvent was removed.
Then water was added, producing a 1 mg/10 µl solution and mixed well.

The obtained solution was dialysed against water at RT overnight, to make sure the solution didn't contain any contaminations or by-products. Therefore a dialysis membrane with a molecular weight cut-off of 2000 Da was used.

4.1.2. Binding DBCO-NHS-Linker to PAMAM

Regarding the linker, a 10 mM solution of DBCO-NHS-Ester in DMSO was used.
It has a molecular weight of 402.4 g/mol and has an absorption maximum at the wavelength of 309 nm (ε_{309} = 11700),
To achieve the conjugation, a reaction of 100 nmol of PAMAM and 1400 nmol of the linker, equalling a fourteen-fold surplus, was set up.
First, 14.3 µl of the 1 mg/10 µl dialysed PAMAM G4 stock solution were mixed with 200 µl 50 mM sodium tetraborate buffer, pH 8.5. Then 140 µl of the 10 mM DBCO-NHS-Linker stock solution was added. The reaction mixture was shaken for 1h at RT. The entire molecular weight of the resulting product, after the NHS-group (115 g/mol) of the linker was cleaved off during the reaction is : PAMAM G4 + DBCO-NHS-Ester = 14502.4 g/mol

After the reaction time, the amount of DBCO-NHS-Linker was determined by measuring the absorbance at 309 nm via Nanodrop.
To remove unbound molecules of the linker, the solution was dialysed against 1xPBS buffer, pH 7.2 overnight at RT, by using a dialysis membrane with a molecular weight cut-off of 2000 Da. The medium was changed twice during the term. Then the amount of the linker (attached to the PAMAM) was checked again and compared to the value before the dialysis.

Before going further to the binding reaction of the protein, the required amount of the PAMAM-DBCO-NHS-Linker solution was freed from any unbound linker molecules using Amicon® centrifugal filter units.
In a typical purification, 100 µl of the dialysed PAMAM-DBCO solution, equivalent to 28 nmol PAMAM, were filled up to 500 µl with 3M guanidine hydrochloride buffer, pH 8.0, then centrifuged in an Amicon® filter for 10 minutes at 10 °C and 12000 rpm. The remaining 100 µl were filled up again and the process was repeated. After each centrifugation step the amount of DBCO-NHS-linker was checked, so the procedure was stopped, when the measured value became nearly constant. It usually took 3 to 4 cycles.

4.1.3. Binding of AhaEc1 to the linker

A 572 µM stock solution of the AhaEc1 protein, which has a MW of 18344.11 g/mol, was used. The amount of protein that was used for the binding reaction was in the same molar relation as PAMAM (ratio 1:1).
As a loss of 10% of PAMAM was expected after the centrifugal filtration, about 25.2 nmol were left (in 140 µl). So 44.03 µl of the protein stock solution, equalling 25.2 nmol, were added and mixed with 44.03 µl of 3M Guanidine hydrochloride. The resulting mixture was shaken at RT at 350 rpm overnight.

4.2. Analytics of the conjugates

4.2.1. SDS-PAGE

To check if the binding reaction was successful, and to define what else was in solution besides the resulting conjugates, a gel electrophoresis was performed on a 12 % polyacrylamide gel. First the separating gel solution was prepared an poured into the gap between the glass plates with 0.75 mm spacers. To receive an even surface of the separating gel, isopropanol was added on top. After 1 h of polymerization, isopropanol was removed and the stacking gel solution was prepared and poured above the separating gel (immediately after addition of APS). A comb with 10 wells was inserted into the stacking gel.
The polymerization was completely achieved after about 1 h.
The gel was then fixed in a Mini Protean 3 cell, in which two gels can be run at the same time. The inner chamber was filled with 1 x cathode buffer and the outer chamber with 1 x anode buffer.

Preparation of the samples:

Before adding Laemmli-buffer, guanidine hydrochloride had to be completely removed from the samples to avoid precipitation. First 200 µl Sephadex - G 50 gel were pipetted into a Mini spin column and the liquid was removed by centrifugation. Then 15 µl of water were added (directly on the gel). After a second spin, the sample was added and the column was spinned down again, so the resulting solution was a purified, guanidine hydrochloride free sample. This process was performed with all guanidine hydrochloride containing samples, prior to gel electrophoresis.

After that, samples containing 1 nmol of conjugates in 10-15 µl were mixed with 10-15 µl Laemmli buffer (max. volume for electrophoresis 30 µl). Then the samples were heated at 95°C for 5 minutes to achieve protein denaturation. Then they were loaded into the wells, after removing the comb, and the gel electrophoresis was run at 150 V, until the front reached the end of the gel (70-80 minutes). The gel was then stained with Coomassie Blue staining solution. After destaining the gel with methanolic acetic acid solution, it was scanned in the densitometer using Quantity One software.

4.2.2. Purification of the conjugates

Considering the result of the gel electrophoresis, PAMAM-DBCO-Ec1 conjugates had to be purified for the upcoming siRNA complexation, to prevent interactions with any unbound molecules (PAMAM-DBCO or free Aha-Ec1).

For this isolation, an *ÄKTA protein purification system* was applied, using the principle of size exclusion chromatography based on gel filtration. All needed programs were initiated via *UNICORN control software*.

First the device was equilibrated with 3M guanidine hydrochloride, pH 8.0 (same buffer as sample) for about 120 minutes.

Then the sample, containing a total of 23.5 nmol of the protein with a volume of 200 µl, was filled up to 500 µl with the same buffer and mixed well as a 500 µl loop was used for sample inlet. After choosing the required program (sample run), the sample was injected, using a 1 ml syringe, and the purification was started. After 120 minutes the chromatography was completed.

The obtained results were customized to display the absorbance only at wavelengths of interest: 260 nm, 280 nm and 309 nm.

Peaks that were too low and obviously did not represent any products were ignored. For every one of the remaining peaks, the associated reported fractions were picked out of the fraction collector and united.

Then the absorbance was measured via Nanodrop. If the determined values were too low, the unified solutions were concentrated using Amicon® centrifugal filter units, receiving volumes of about 100 µl. A second measurement was done after concentrating, to calculate the amount of protein contained in every solution and consider the form of the absorbance curve.
To detect the right value of protein in any of the solutions containing conjugates of PAMAM-DBCO-Ec1, the following formula was used to calculate the absorbance:

$$A = A_{280} - (A_{309} \times 1.089) \quad \text{and} \quad \varepsilon = 15470$$

4.2.3. Gel-analysis of conjugates

To find out, which of the collected solutions exclusively contained conjugates of PAMAM-DBCO-Ec1, another gel electrophoresis with a 12 % polyacrylamide gel was performed, as described above, including the washing step using Mini spin columns.

4.3. siRNA complexation

After specification of isolated conjugates via gel analysis, the siRNA complexation was carried out. Since the binding forces in this reaction were ionic interactions, the required amounts of conjugates and siRNA were calculated as follows:
PAMAM G4 has a total of 64 positively charged amino groups, while the used double stranded siRNA has 41 negatively charged phosphate groups. So 3 samples with PAMAM to siRNA, respectively amino to phosphate (N/P) ratios of : 0.5 , 1 and 2 were prepared.

N in PAMAM	64
P in siRNA	41
N/P ratio	1.56
MW	46 348 g/mol

N/P ratio	molar ratio	siRNA (nmol)	Protein (nmol)	µl siRNA (58.5 µM)	µl Conjugate (126.3 µM)	Sample	total volume (µl)
0.5	0.32	3.00	0.97	51.30	7.68	1	58.9
1	0.64	2.85	1.90	48.71	15.04	2	63.8
2	1.28	2.30	3.00	39.30	23.69	3	62.9

As the prepared solutions were still containing denaturating guanidine hydrochloride, they had to be rebuffered, so that the complexation could actually proceed.
Therefore, all samples were dialysed against 1x PBS buffer, pH 7.2 at RT overnight, by using a dialysis membrane with a molecular weight cut-off of 2000 Da. The buffer was exchanged twice.

Due to the importance of receiving guanidine hydrochloride free samples following precautions were carried out:

- The absorbance was measured via Nanodrop, especially considering the form of the curve as an indication of solubility
- The pH level was measured and set to 7.2, if the sample was totally dissolved in PBS buffer
- Small volumes of the samples (10 µl) were added to 10-15 µl of Laemmli buffer, again to check the solubility: In case guanidine hydrochloride was still present, precipitation would occur immediately

Furthermore the amount of siRNA was determined by using the absorbance of the Nanodrop measurement, and compared to the applied amount before the samples were dialysed. siRNA (the used duplex) absorbs at 260 nm (ε_{260} = 414300).

4.3.1. Gel electrophoresis

A 10 % polyacrylamide gel solution in 10 x TBE buffer was prepared and poured between two glass plates with 0.75 mm spacers. Then a comb was inserted and it was left for polymerization. The gel was pre run at 4°C and 100 V for 30 minutes.

Before starting the electrophoresis, all samples were incubated for 30 minutes at RT to guarantee complete complexation. Volumes equalling 0.5 noml siRNA were used from each of the three samples (N/P = 0.5, 1 and 2).

In addition, a sample containing 0.5 nmol of the applied double stranded siRNA (58.5 µM) was prepared. All samples were finally prepared adding DNA loading dye up to a maximum of 25 µl.

Sample	N/P ratio	Sample (µl)	DNA loading dye (µl)
0	0	8.5	15.5
1	0.5	9.8	14.5
2	1	11.2	13.0
3	2	14.7	9.0

After removing the comb, the samples were loaded into the wells. In the first well, 5 µl of DNA loading dye were added to enable tracking of the migration distance. The tank was filled with 1 x TBE buffer and the gel was run at 4°C and 100 V for nearly 80 minutes. At the end of the electrophoresis, bromophenol blue had migrated to the lower third of the gel, while xylene cyanol remained in the middle. Then the gel was stained with methylene blue solution and destained by repeated rinsing (3x 10 min) with water. The destained gel was scanned in the densitometer. The resulting bands were quantified by using Quantity One software.

4.4. Cell culture

Two cell lines were used:
1. HeLa (cervical cancer cells) and
2. MDA-MB 468 (Human breast cancer cells)
Both cell lines were cultivated in DMEM complemented with 10 % foetal bovine serum without any antibiotics.
For splitting, the cells were washed with PBS after removing the medium from the flasks. Then PBS was removed and the cells were trypsinized using trypsin solution. The detached cells were then resuspended in DMEM and grown in the incubator at 37 °C, 5% CO_2.

4.4.1. Luciferase Assay

The entire assay was carried out within five days. On the first day, after splitting the cells, they were seeded in two 96-well plates, one for each cell line and incubated overnight at 37 °C, 5% CO_2. In 90 µl per well 20.000 MDA-MB-468 cells for the first plate, and 15.000 HeLa cells for the other one were required.

Therefore concentrations of the cell suspensions were determined, using a counting chamber, and dilutions with the needed amounts were produced. :

Cell line	Cells needed / well	Cells needed / ml	cells / ml (counted)	Dilutions *
MDA-MB-468	20.000	222 222	1 170 000	1 : 5.26
HeLa	15.000	166 666	1 170 000	1 : 7.02

* Dilutions were produced by adding 4.26, respectively 6.02 parts of Opti-MEM®-DMEM to 1 part of the cell suspensions

On the second day, both cell lines were transfected with plasmid DNA. Therefore dilutions of pEGFPLuc (pDNA A) and pCMV-Gluc (pDNA B) were mixed with Opti-MEM®-DMEM, producing concentrations of 100 ng/µl of each component. Lipofectamine™ 2000 was used for transfection, after diluting it with an Opti-MEM®-DMEM mixture. Then Lipofectamine was added to the diluted pDNA and the mixtures were transferred to the wells, except for controls, where the cells were left without treatment. So each well with treated cells contained:

- ⇨ 20.000 MDA- or 15.000 HeLa cells
- ⇨ 2.5 µl of pDNA A+B (100ng/µl each) and
- ⇨ 0.3 µl Lipofectamine

After 24 h of incubation, the cells were treated with the samples containing PAMAM-Ec1-siRNA conjugates or only PAMAM and siRNA without protein. Thus, stock solutions of the samples were diluted with Opti-MEM®, producing volumes of 10 µl of for each well with either 50 or 100 pmol siRNA for each sample:

Sample	pmol siRNA	sample stock concentrations [µM]	volume/well [µl]	Volume Stock [µl]	Volume Opti-MEM® [µl]
PAMAM-Ec1-siRNA N/P 0.5	100	50.8	20	19.69	180.31
PAMAM-Ec1-siRNA N/P 1	100	23.5	20	42.55	157.45
PAMAM-Ec1-siRNA N/P2	100	36.5	20	27.40	172.60
PAMAM-siRNA N/P 0.5	100	58.6	20	17.06	182.94
PAMAM-siRNA N/P 1	100	54.9	20	18.21	181.79
PAMAM-siRNA N/P 2	100	48.7	20	20.53	179.47

For 50 pmol/well 70 µl of each above prepared sample were diluted with 70 µl Opti-MEM®. As a positive control, 1 pmol Anti-Luciferase siRNA duplex was applied with lipofectamine. Therefore, a siRNA stock solution was first mixed, again with Opti-MEM®. Then a mixture of Lipofectamine® RNAiMAX and Opti-MEM®(a) was incubated and added to a siRNA-Opti-MEM mixture (b) :

		b		a	
pmol siRNA	volume/well	siRNA 62.9 µM total volume [µl]	Volume Opti-MEM® [µl]	Lipofectamine [total volume]	Volume Opti-MEM® [µl]
10	20	1,59	84,21	30	84,21

All samples were applied in triplicates of wells in both plates after adding medium to all wells. Finally the cells were incubated at 37 °C, 5% CO_2 for 48 h.
On the last day the luminescence intensity was detected to determine the transfection efficiency. First the medium was removed from all wells and transferred to new plates. Each of these plates was inserted into the plate reader (Infinite M 200 PRO). The injector was set to add 50 µl of Stop & Glo Reagent to each well, containing a substrate for Gaussia Luciferase (coelenterazine), which enabled the determination of its luminescence signal. Then the cells in the original plates were washed with 100 µl PBS. After adding 20µl lysis buffer to each well, the plates were shaken for 30 minutes to completely lyse the cells. Before determining the luminescence signal caused by the Firefly luciferase - catalysed reaction the Injector added 50 µl of LAR II (luciferin).

MDA-MB-468 (Plate 1)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*
B	*untreated*	pDNA A+B	50 pmol Ec1-PAMAM conjugate N/P 0.5	50 pmol Ec1-PAMAM conjugate N/P 0.5	50 pmol Ec1-PAMAM conjugate N/P 0.5	pDNA A+B	50 pmol PAMAM/siRNA N/P 0.5	50 pmol PAMAM/siRNA N/P 0.5	50 pmol PAMAM/siRNA N/P 0.5	pDNA A+B	pDNA A+B	*untreated*
C	*untreated*	pDNA A+B	50 pmol Ec1-PAMAM conjugate N/P 1	50 pmol Ec1-PAMAM conjugate N/P 1	50 pmol Ec1-PAMAM conjugate N/P 1	pDNA A+B	50 pmol PAMAM/siRNA N/P 1	50 pmol PAMAM/siRNA N/P 1	50 pmol PAMAM/siRNA N/P 1	pDNA A+B	pDNA A+B	*untreated*
D	*untreated*	pDNA A+B	50 pmol Ec1-PAMAM conjugate N/P 2	50 pmol Ec1-PAMAM conjugate N/P 2	50 pmol Ec1-PAMAM conjugate N/P 2	pDNA A+B	50 pmol PAMAM/siRNA conjugate N/P 2	50 pmol PAMAM/siRNA conjugate N/P 2	50 pmol PAMAM/siRNA conjugate N/P 2	pDNA A+B	pDNA A+B	*untreated*
E	*untreated*	pDNA A+B	100 pmol Ec1-PAMAM conjugate N/P 0.5	100 pmol Ec1-PAMAM conjugate N/P 0.5	100 pmol Ec1-PAMAM conjugate N/P 0.5	pDNA A+B	100 pmol PAMAM/siRNA N/P 0.5	100 pmol PAMAM/siRNA N/P 0.5	100 pmol PAMAM/siRNA N/P 0.5	pDNA A+B	pDNA A+B	*untreated*
F	*untreated*	pDNA A+B	100 pmol Ec1-PAMAM conjugate N/P 1	100 pmol Ec1-PAMAM conjugate N/P 1	100 pmol Ec1-PAMAM conjugate N/P 1	pDNA A+B	100 pmol PAMAM/siRNA N/P 1	100 pmol PAMAM/siRNA N/P 1	100 pmol PAMAM/siRNA N/P 1	pDNA A+B	pDNA A+B	*untreated*
G	*untreated*	pDNA A+B	100 pmol Ec1-PAMAM conjugate N/P 2	100 pmol Ec1-PAMAM conjugate N/P 2	100 pmol Ec1-PAMAM conjugate N/P 2	pDNA A+B	100 pmol PAMAM/siRNA N/P 2	100 pmol PAMAM/siRNA N/P 2	100 pmol PAMAM/siRNA N/P 2	pDNA A+B	pDNA A+B	*untreated*
H	*untreated*	pDNA A+B	**1 pmol siRNA+LF**	**1 pmol siRNA+LF**	**1 pmol siRNA+LF**	*untreated*	**10 pmol siRNA+LF**	**10 pmol siRNA+LF**	**10 pmol siRNA+LF**	pDNA A+B	pDNA A+B	*untreated*

HeLa (Plate 2)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*	*untreated*
B	*untreated*	pDNA A+B	50 pmol Ec1-PAMAM conjugate N/P 0.5	50 pmol Ec1-PAMAM conjugate N/P 0.5	50 pmol Ec1-PAMAM conjugate N/P 0.5	pDNA A+B	50 pmol PAMAM/siRNA N/P 0.5	50 pmol PAMAM/siRNA N/P 0.5	50 pmol PAMAM/siRNA N/P 0.5	pDNA A+B	pDNA A+B	*untreated*
C	*untreated*	pDNA A+B	50 pmol Ec1-PAMAM conjugate N/P 1	50 pmol Ec1-PAMAM conjugate N/P 1	50 pmol Ec1-PAMAM conjugate N/P 1	pDNA A+B	50 pmol PAMAM/siRNA N/P 1	50 pmol PAMAM/siRNA N/P 1	50 pmol PAMAM/siRNA N/P 1	pDNA A+B	pDNA A+B	*untreated*
D	*untreated*	pDNA A+B	50 pmol Ec1-PAMAM conjugate N/P 2	50 pmol Ec1-PAMAM conjugate N/P 2	50 pmol Ec1-PAMAM conjugate N/P 2	pDNA A+B	50 pmol PAMAM/siRNA conjugate N/P 2	50 pmol PAMAM/siRNA conjugate N/P 2	50 pmol PAMAM/siRNA conjugate N/P 2	pDNA A+B	pDNA A+B	*untreated*
E	*untreated*	pDNA A+B	100 pmol Ec1-PAMAM conjugate N/P 0.5	100 pmol Ec1-PAMAM conjugate N/P 0.5	100 pmol Ec1-PAMAM conjugate N/P 0.5	pDNA A+B	100 pmol PAMAM/siRNA N/P 0.5	100 pmol PAMAM/siRNA N/P 0.5	100 pmol PAMAM/siRNA N/P 0.5	pDNA A+B	pDNA A+B	*untreated*
F	*untreated*	pDNA A+B	100 pmol Ec1-PAMAM conjugate N/P 1	100 pmol Ec1-PAMAM conjugate N/P 1	100 pmol Ec1-PAMAM conjugate N/P 1	pDNA A+B	100 pmol PAMAM/siRNA N/P 1	100 pmol PAMAM/siRNA N/P 1	100 pmol PAMAM/siRNA N/P 1	pDNA A+B	pDNA A+B	*untreated*
G	*untreated*	pDNA A+B	100 pmol Ec1-PAMAM conjugate N/P 2	100 pmol Ec1-PAMAM conjugate N/P 2	100 pmol Ec1-PAMAM conjugate N/P 2	pDNA A+B	100 pmol PAMAM/siRNA N/P 2	100 pmol PAMAM/siRNA N/P 2	100 pmol PAMAM/siRNA N/P 2	pDNA A+B	pDNA A+B	*untreated*
H	*untreated*	pDNA A+B	**1 pmol siRNA+LF**	**1 pmol siRNA+LF**	**1 pmol siRNA+LF**	*untreated*	**10 pmol siRNA+LF**	**10 pmol siRNA+LF**	**10 pmol siRNA+LF**	pDNA A+B	pDNA A+B	*untreated*

4.4.2 Binding of fluorescent siRNA to EpCAM-positive and –negative cells

A siRNA duplex was produced using Luc siRNA AS Alexa fluor 568 and SS. Equimolar amounts of both strands were mixed and incubated at 80°C for 10 minutes, then cooled down to 4°C on ice.

Then PAMAM-Ec1-conjugates were complexed to this siRNA duplex in the same N/P ratios produced for the Luciferase Luminescence Assay (N/P = 0.5 / 1 / 2). Again, the same ratios were produced with PAMAM only instead of the protein conjugates. The obtained samples were dialysed overnight at 25 °C using dialysis membrane with a molecular weight cut-off of 2000 Da against 1 x PBS buffer, pH 7.2.

For the flow cytometry analysis, two cell lines, MDA-MB-468 and HeLa cells were first trypsinized with 1 ml trypsin each at 37°C for 3-5 minutes, then resuspended in PBS (incl. 1 % FCS). Using a counting chamber, the cells were counted in order to produce dilutions complying with the required amount of cells per eppi, 180.000 cells in 135 µl. The cells were washed with 3 ml PBS, transferred into a Falcon tube and centrifuged. After removing the supernatant, they were resuspended in PBS (incl. 1 % FCS).

Then the samples were prepared by diluting the stock solutions. Each cell line was treated with each of the three N/P ratios, with and without AhaEc1. Moreover untreated MDA-MB-468 and HeLa cells were seeded. Also in two tubes, the cells were transfected with naked Luciferase Alexa fluor 568 Antisense siRNA, one of each line. For each sample, amounts equivalent to 500 pmol siRNA were applied.
The required amounts of the diluted samples were added to the cells and the mixtures were incubated on ice for 1h. Then each mixture was washed with 1 ml PBS (incl. 1 % FCS), centrifuged and the supernatant was removed. The washing step was repeated once, and 500 µl ice cold PBS (incl. 1 % FCS) was added to each tube. Finally the solutions were transferred into FACS tubes and stored on ice.

Samples labeled with Alexa fluor 568
500 pmol N/P 0.5 MDA-MB-468
500 pmol N/P 1 MDA-MB-468
500 pmol N/P 2 MDA-MB-468
500 pmol N/P 0.5 HeLa
500 pmol N/P 1 HeLa
500 pmol N/P 2 HeLa
500 pmol PAMAM-siRNA N/P 0.5 MDA-MB-468
500 pmol PAMAM-siRNA N/P 1 MDA-MB-468
500 pmol PAMAM-siRNA N/P 2 MDA-MB-468
500 pmol PAMAM-siRNA N/P 0.5 HeLa
500 pmol PAMAM-siRNA N/P 1 HeLa
500 pmol PAMAM-siRNA N/P 2 HeLa
untreated cells HeLa
untreated cells MDA-MB-468
Luc siRNA AS Alexa fluor 568 HeLa
Luc siRNA AS Alexa fluor 568 MDA-MB-468

To analyse the cells, the tubes were inserted into the instrument (BD FACS Calibur™). 10.000 cells of each sample were analysed, and a histogram of counts for the respective fluorescence channel (FL2-H) was generated. For comparison between samples, the geometric means were used.

5. Results and Discussion

To achieve successful synthesis of siRNA-PAMAM-Ec1 conjugates to specifically target EpCAM positive cells, different coupling strategies and analytic methods were used. While the DARPin conveys receptor-specific uptake, PAMAM offers the ability to bind a large amount of siRNA per molecule, facilitated by the high number of positive charges.
Thus, instead of conventional chemical conjugation of siRNA to the receptor with a 1:1 ratio, a higher quantity of the gene downregulating nucleic acid can enter the cells with each receptor uptake event. Additionally, the basic dendrimer is designed to facilitate endosomal escape in order to enable the siRNA-guided influence on gene expression.

5.1. Conjugation of DARPin to the dendrimer

Conjugation of Linker to dendrimer

For the first reaction, the attachment of DBCO-NHS-ester to the PAMAM G4 dendrimer, a click chemistry strategy was applied. Through an active ester reaction, DBCO - linker was linked to the amino surface groups of the dendrimer producing an amide bond.
The aim was to generate conjugates with an equimolar ratio of DARPin and dendrimer. The NHS ester reaction proceeds in lower yield than the click chemistry strategy, which usually achieves nearly quantitative yields. Therefore, the strategy was to attach a higher amount of linker to the dendrimer than would be used for protein attachment. The remaining linkers (unreacted with protein) would not interfere with siRNA complexation or cellular uptake.

A fourteen fold surplus of the linker was applied, because this reaction runs slowly and the NHS group is hydrolysed in aqueous solutions as a competing reaction.
After the reaction, unbound DBCO linker molecules were removed by dialysis with a molecular weight cut-off of 2 kDa. (MW of DBCO-NHS-ester = 402.4 g/mol). It was interesting to find out the ratio between PAMAM and DBCO after the reaction and later after the dialysis to know the amount of the linker, that would remain bound to PAMAM.

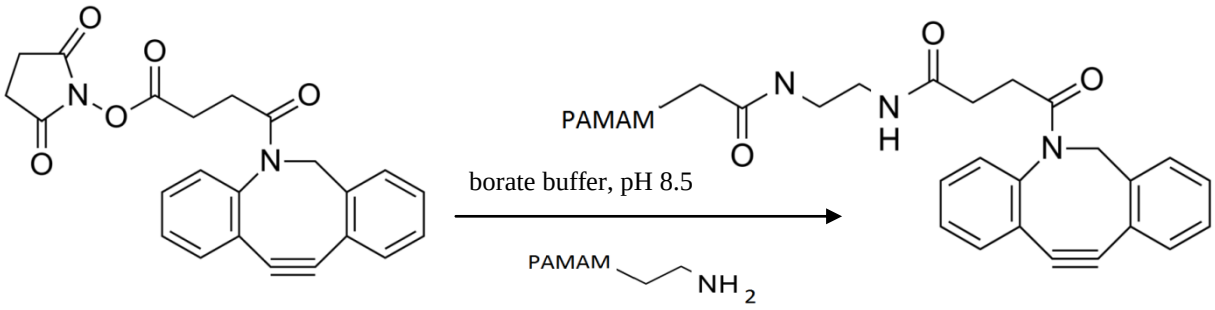


Figure 6: Conjugation of PAMAM and DBCO

The first challenge was to determine the amount of the dendrimer during the entire work. PAMAM G4 has a known molecular weight (14215 g/mol), but neither the extinction coefficient, nor the exact wavelength of absorbance are detectable. However, prior UV-vis spectroscopic studies have shown, that PAMAM has an absorption band with λ_{max} between 280 and 285 nm, but the appearance of the band is directly dependant on the pH and the corresponding protonation of the amines. [35]

Several trials were performed in order to define the extinction coefficient with an exact knowledge of the used amount using Nanodrop. Unfortunately, the measurements were not conclusive, as a lot of different values resulted. Another measurement was done via UV-spectrophotometer, but again there were no meaningful results. As a last try, a Bradford Assay was carried out using different concentrations of the dendrimer and BSA as a standard, hoping to receive a linear absorbance curve, but once more the results were highly variable.

Anyhow, as there was no exact method to measure the dendrimer, we used for all upcoming calculations the same quantity of PAMAM that was used at the beginning of the reaction. The molecular weight cut-off of the dialysis membrane was 2000 Da and the MW of PAMAM is much higher, it certainly remains in the stock solution.

The accurate extinction coefficient (ε_{309} = 11700) of DBCO-NHS-ester was self-determined by producing and measuring a 1:100 dilution of the available 10 mM DMSO solution in 5 x PBS,. The absorbance spectrum shows significant bonds at 280 nm and 309 nm; the ratio A_{280}/A_{309} is 1.089, which was used for subsequent calculations.

The reaction was performed in 50 mM sodium tetraborate buffer, pH 8.5 with different amounts of DBCO to check the ratio of PAMAM to the linker under diverse conditions. Example of the results of reactions with different surpluses of DBCO-NHS-ester compared to PAMAM, after dialysis:

DBCO - Surplus	Ratio PAMAM : DBCO
7x	1 : 0.71
14x	1 : 7.2
28x	1 : 7.4

Table 1: PAMAM-DBCO ratios after dialysis

So there is a significant difference between a seven- and a fourteen-fold surplus. A ratio of 1 : 0.71 was insufficient to achieve a 1:1 conjugation of protein to PAMAM. However, the ratios of a 14x and a 28x surplus were almost equal. Therefore a fourteen-fold surplus of DBCO was used.

After performing the binding reaction to the protein (see later) a few times with the produced solution of PAMAM-DBCO and analysing the products by gel electrophoresis, there were significant bands representing unbound PAMAM-DBCO and protein in a high proportion. So there were suspicions, that the unbound molecules of the linker were not completely removed by dialysis. The result could be a competition between PAMAM-DBCO conjugates and free DBCO molecules for the binding sites of the protein. Thus the unwanted product DBCO-Ec1 would possibly arise.

For this reason the additional purification with Amicon®-filters was carried out and the result was as expected: Instead of the PAMAM : DBCO ratio of 1 : 7.2 , it was only 1 : 3.3 after centrifugal filtration (three times), whereas the amount of DBCO decreased with each step. A 10 % loss of PAMAM in consequence of this method was included in the calculation.

	before centrifugal filtration	after 1st centrifugation	after 2nd centrifugation	after 3rd centrifugation
PAMAM : DBCO Ratio	1 : 7.2	1 : 4	1 : 3.4	1 : 3.3

Table 2: PAMAM-DBCO ratios during and after centrifugal filtration

Binding of AhaEc1 to the linker

Generally, a variety of coupling chemistries could be applied for this reaction. The most commonly used strategy is the *malemide-thiol coupling* for reactions between thiol containing proteins and malemide groups of linkers. The major disadvantage of this method is that the reaction is generally not site-specific, especially when naturally existing reactive groups of protein ligands are used. Increasing protein size complies with a rising number of reactive functionalities, such as amines, thiols, carboxylic and acidic groups, which may lead to heterogenous or dysfunctional conjugates.

A more suited method for achieving site specific coupling is Cu(I)-catalyzed click chemistry, such as the *azide-alkyne [3+2] cycloaddition (CuAAC).* However, using copper catalyst represents a limit for the reaction due to the instability of Cu(I) and its cytotoxic effects. The attachment of Cu(I) to proteins makes a complete removal of the catalyst very challenging. To overcome these disadvantages, Cu-free click chemistry has been recently developed, for example the the *strain-promoted azido-alkyne cycloaddition (SPAAC)*, which was used in this work. In this case, the DARPin Ec1 was modified by the substitution of the N-capping methionine by azidohomoalanine (Aha) producing a clickable protein which enables a high yielding site-specific reaction between azides and cyclooctynes under mild conditions. As the ring-strained alkyne shows high reactivity, the addition is carried out spontaneously and the use of a metal catalyst is not necessary. [36, 37]

Regarding cooper induced toxic effects on cells, especially concerning the damage of cell membranes, and protein interactions which have been reported in previous studies, this type of reaction is of great benefit. [38, 39]

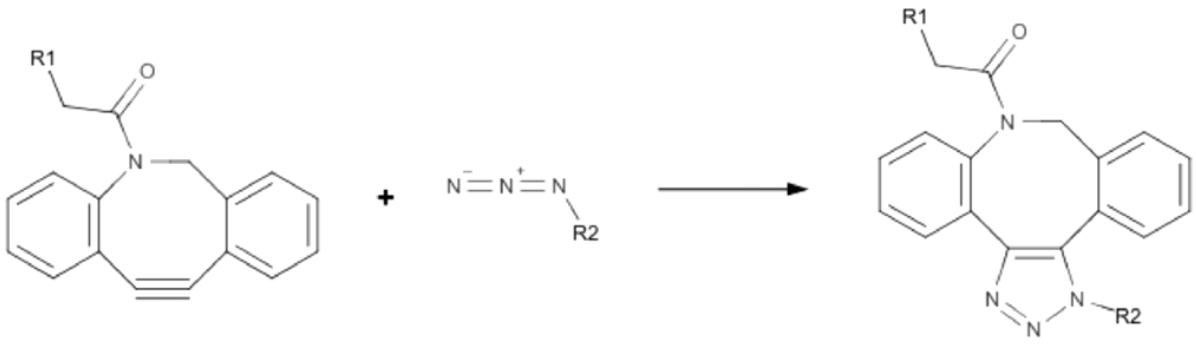


Figure 7: Cycloaddition between dibenzocyclooctyl and azide

At the beginning, the reaction was performed several times using different buffers, especially PBS (pH 7.2) and $Na_2B_4O_7$ (pH 8.5). We used the same molar amount of the Aha-Ec1-Protein as PAMAM G4. After the reaction time of 24h, the amount of protein in the solution was measured via Nanodrop. However, the determined value was much lower than the actually used amount. Also, a SDS-PAGE of this reaction demonstrated, that almost no conjugates were generated, and the solution only contained free protein and unreacted PAMAM-DBCO.

So concerning these two buffers, the reaction seemed to be not completely successful. The only possible explanation for this loss was, that either the protein itself, the resulting conjugates, or both were not soluble in PBS or $Na_2B_4O_7$ buffer. To check, if this was the case, an Eppi with the reaction solution was centrifuged for 10 minutes after the amount of soluble protein was determined. Then the supernatant was removed and the insoluble residue was resolved in different buffers, including 3M guanidine hydrochloride (pH 8.0).
In the denaturing solvent, a significant amount of protein was detected in the form of free protein and weak bands of conjugates. Clearly, a large proportion of the AhaEc1 protein precipitated in the PBS reaction solution.

So we finally decided to use 3M GuHCl for this reaction to achieve adequate yields, despite the denaturation, which could arise difficulties for the upcoming siRNA complexation and the following cell culture experiments.

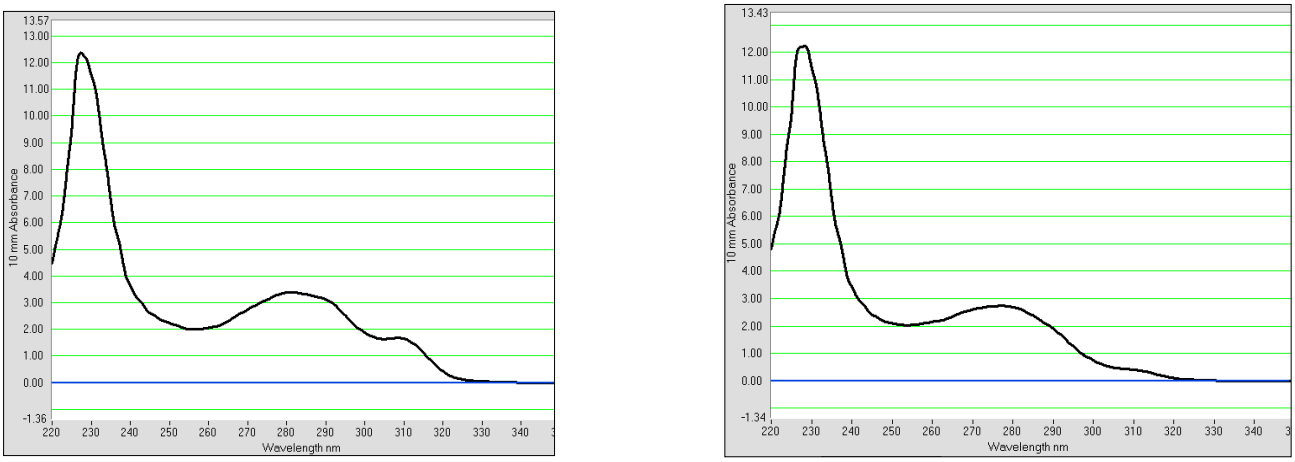
As previously mentioned, concentrations of the products after the reactions were determined by spectrophotometric measurements. The maximal absorbance of AhaEc1 itself is at 280 nm with the coefficient $\varepsilon_{280} = 15470\ \ l \cdot mol^{-1} \cdot cm^{-1}$.

However the linker interferes with the protein absorbance at the same wavelength, unlike the dendrimer. So for determination of protein concentrations, the values for both wavelengths 280 nm and 309 nm were considered, and the calculation included the deduction of the absorbance of the linker, having regard to the previously defined ratio (1.089). So protein concentrations were detected as follows:

$$A = A_{280} - (A_{309} \times 1.089) \quad \text{and} \quad \varepsilon = 15470$$

	Volume (µl)	A280 (Protein+Linker)	A_{309} (Linker)	A_{280} (Protein)	C (Protein) (µmol/l)	Protein amount (nmol)
Before conjugation	228	3.396	1.706	1.690	109.6	25.2
After 24 h	179	2.698	0.520	2.178	140.8	25

Table 3: Example for spectrophotometric analysis and calculation of protein concentrations



Before conjugation

After conjugation

Figure 8: Absorption spectrums before and after reacting time

5.2. Identification and analyses of the conjugates

Gel electrophoresis

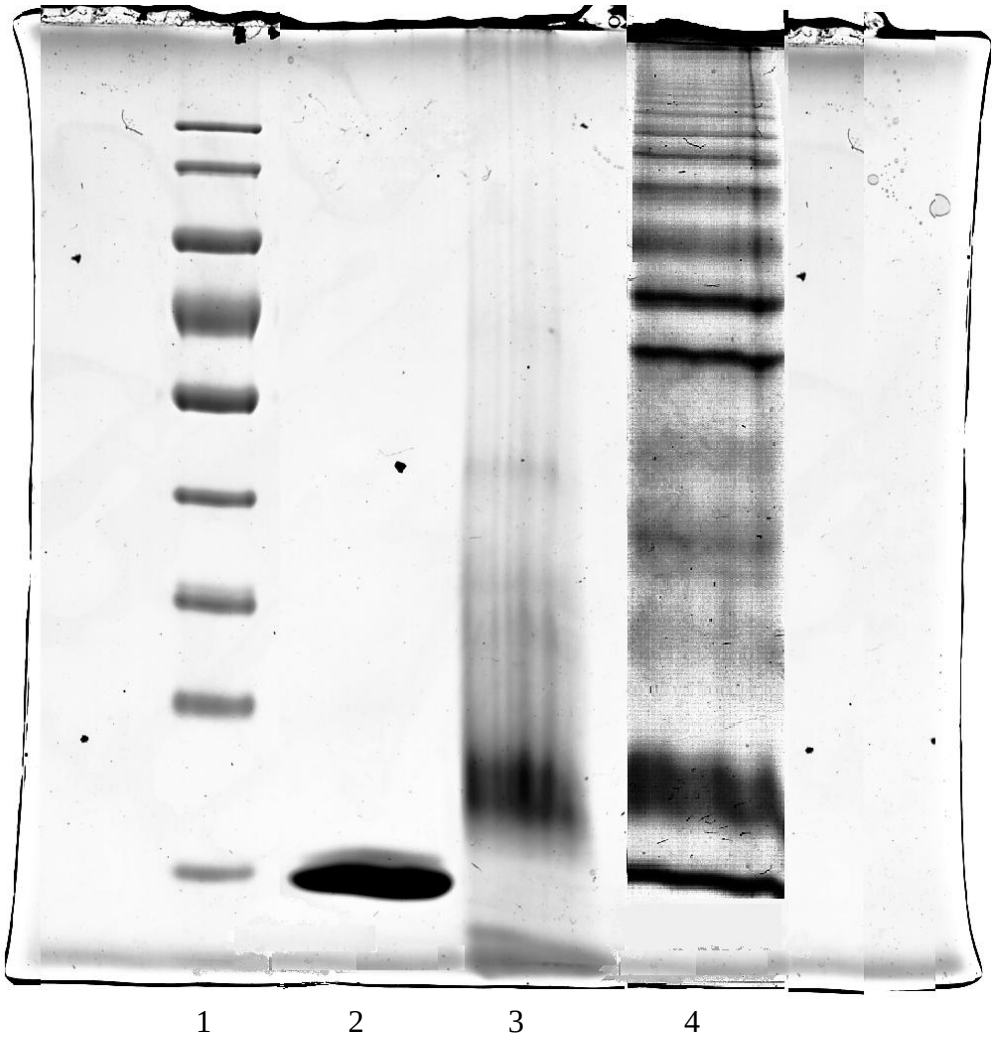
For a long time polyacrylamide gel electrophoresis has been known as an effective, rapid and easy technique for separating, identifying and analysing polyamidoamine compounds. Moreover, using Coomassie Blue dye for staining the gel has been described as the most precise way to receive a significant visualisation of PAMAM and proteins. [40-42]

Despite a few obstacles at the beginning, PAGE was indeed proven to be very useful to identify the obtained conjugates after the protein binding reaction. Otherwise there would be no evidence for the success of the reaction, or if the whole amount of protein was actually used for producing the intended conjugations. It took some attempts to find out, that a volume equalling 1 nmol of AhaEc1, was enough to have a clear separation in all samples containing protein.

As for the dendrimer itself, we used 10 µl of the PAMAM-DBCO solution (after dialysis), equalling 2 nmol PAMAM for comparison. There were some difficulties at first, as the site of the gel, where PAMAM was applied, was often smeared after destaining and did not show any clear bands. Anyway, it usually ran well, when 2 nmol were used.

An exact allocation of conjugates, free protein, and unreacted PAMAM-DBCO was possible by using a protein ladder standard.

An important step before transferring the samples to the gel was desalting to remove GuHCl. Both, Laemmli buffer, which was added to all samples, and the gel itself contain SDS and this leads to immediate precipitation in conjunction with GuHCl. The purification with Sephadex gel in Mini spin columns using water, was well suited, as there was no loss of the applied volume, after repeated spin down steps.



Gel A

1. PageRuler Standard (4µl)
2. AhaEc1
3. PAMAM-DBCO after dialysis (2nmol)
4. Conjugate after protein conjugation

Figure 9: Gel electrophoresis of Ec1, PAMAM-DBCO and the conjugates
12% SDS-polyacrylamide gel - 1nmol of each sample was applied , if not indicated otherwise

As shown in figure 9, the gel analysis demonstrated very conclusive results with a clear evidence of the components of the obtained product solution.
The sample obviously contained conjugates on the one hand, and unbound protein and PAMAM-DBCO on the other hand, which is an indication that the reaction was successful, but the protein was not completely consumed for the conjugation.

Moreover, the conjugates show a variety of molecular weights, indicating a distribution of conjugates with increasing number of DARPin per dendrimer.
According to the used standard, only weak bands are visible in the range of 30.000 Da, where the expected product with a 1:1 molar ratio to the protein (MW=32846 g/mol) is expected. However, there are more significant bands apparent starting at 70.000 Da and raising up to 130.000 Da.
So, clearly, the major part of the received conjugates seems to consist of one molecule of PAMAM linked to, not one, but three, four, five or six molecules of the DARPin. However, the migration of PAMAM and its conjugates do not necessarily correspond to the protein ladder due to the differing chemical structure and their multiple cationic charges. Considering the fact, that siRNA binds to PAMAM and not to Ahac1, the number of protein molecules is of minor importance for the use as siRNA delivery system.

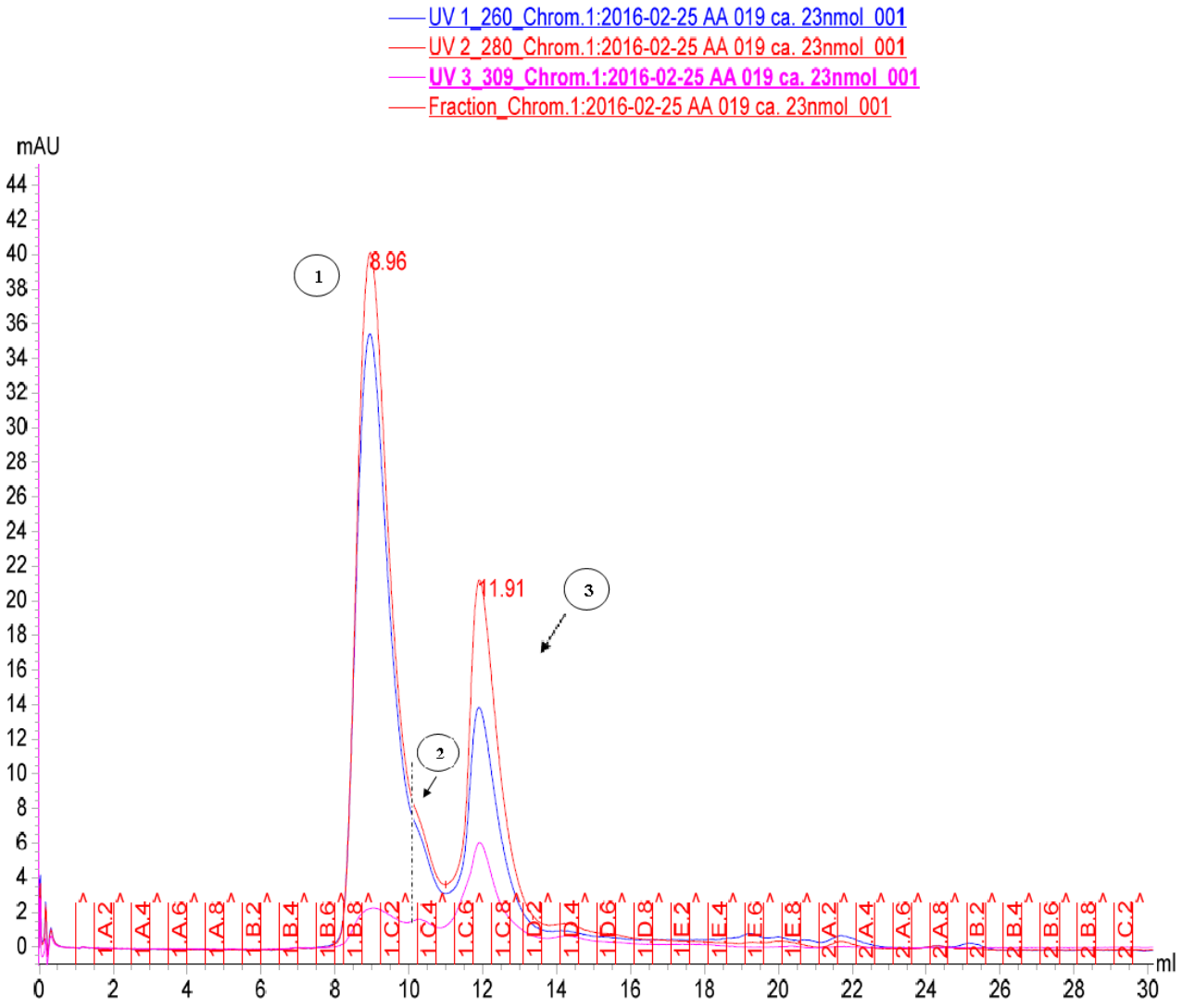
x	1	2	3	4	5	6
MW (g/mol)	32 846	51 190	69 534	87 878	106 222	124 566

Table 4: Molecular weights of conjugates of 1 mol PAMAM and x mol AhaEc1

The outstanding question remained, what proportion of the used protein had actually reacted.

Isolation of the conjugates by ÄKTA
As a matter of fact, for specific receptor-mediated binding and uptake, each PAMAM molecule should carry at least one protein.. Unreacted PAMAM educts would hamper specific binding and uptake. In order to find an appropriate method to isolate the conjugates, a size exclusion chromatography seemed to be the best option. First a trial with a benchtop Sephadex Gel column purification was carried out, but was proven to be complicated, as the separation between protein, PAMAM, and conjugates was insufficient.

However, the ÄKTA protein purification system turned out to be an efficient and simple way to achieve the isolation, using the principle of a gel permeation chromatography. After separating all components of the inserted sample the results shown as peaks of absorbance at different wavelengths, enabling an explicit identification of the molecules.



No	Peak name	Retention	Area	Height
		ml	ml*mAU	mAU
1	-	-0.03	0.0932	16.734
2	-	0.03	1.7548	20.213
3	-	0.17	2.3261	18.424
4	-	0.31	45.0460	16.659

Figure 10: An example of results of ÄKTA purification

As evident in Figure 10, the system separated three different products, each represented by a peak, considering peak 2 as a separate compound, as is does not show the same gradient as peak 1. The result also shows the retention volume, the height of the signal and the position of the corresponding fractions in the fraction collector. So the displayed fractions belonging to each peak were collected separately and united producing three solutions:

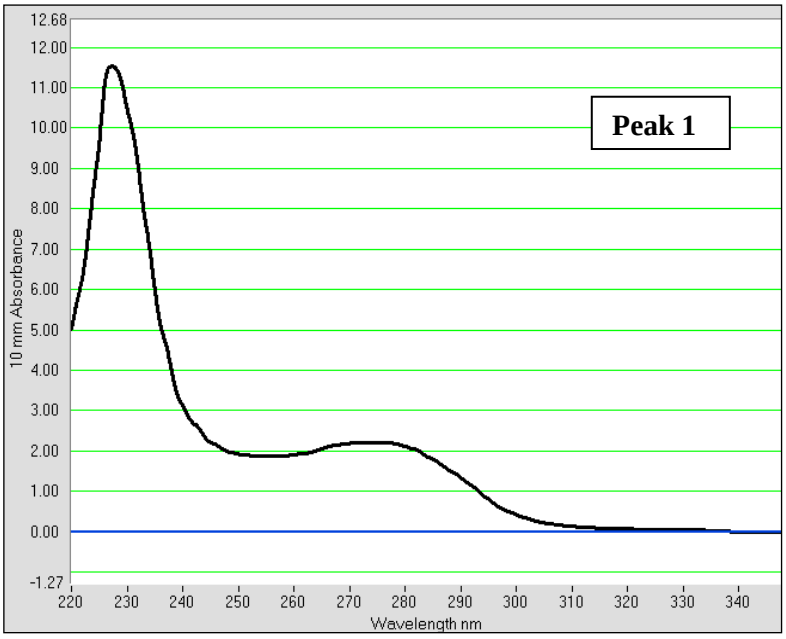
Peak	collected fractions	Absorbance at ()
1	1B7; 1B8; 1C1; 1C2	260 nm; 280 nm
2	1C3; 1C4	260 nm; 280 nm
3	1C6; 1C7; 1C8	260 nm; 280 nm; 309 nm

Table 5: Fractions collected for each isolated compound

After concentrating the samples via centrifugal filtration, the absorbance of the solutions was measured via Nanodrop to determine the amounts of protein for each product. The inserted solution contained 23.5 nmol of protein:

Sample (Peak)	Protein (nmol)	Percentage
1	12.63	53.7 %
2	2.58	10.9 %
3	5.48	23.3 %
Total	20.69	88.0 %

Table 6: Protein amounts in collected solutions



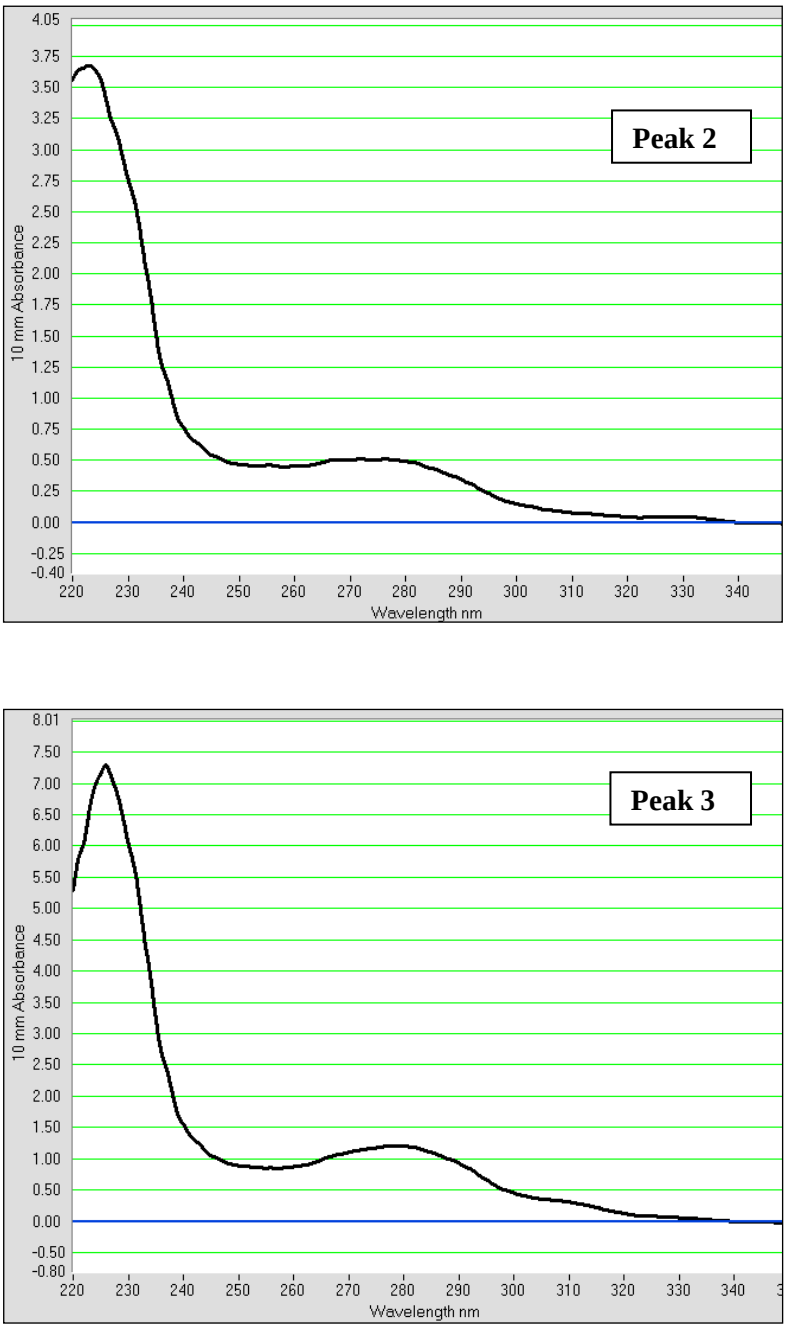
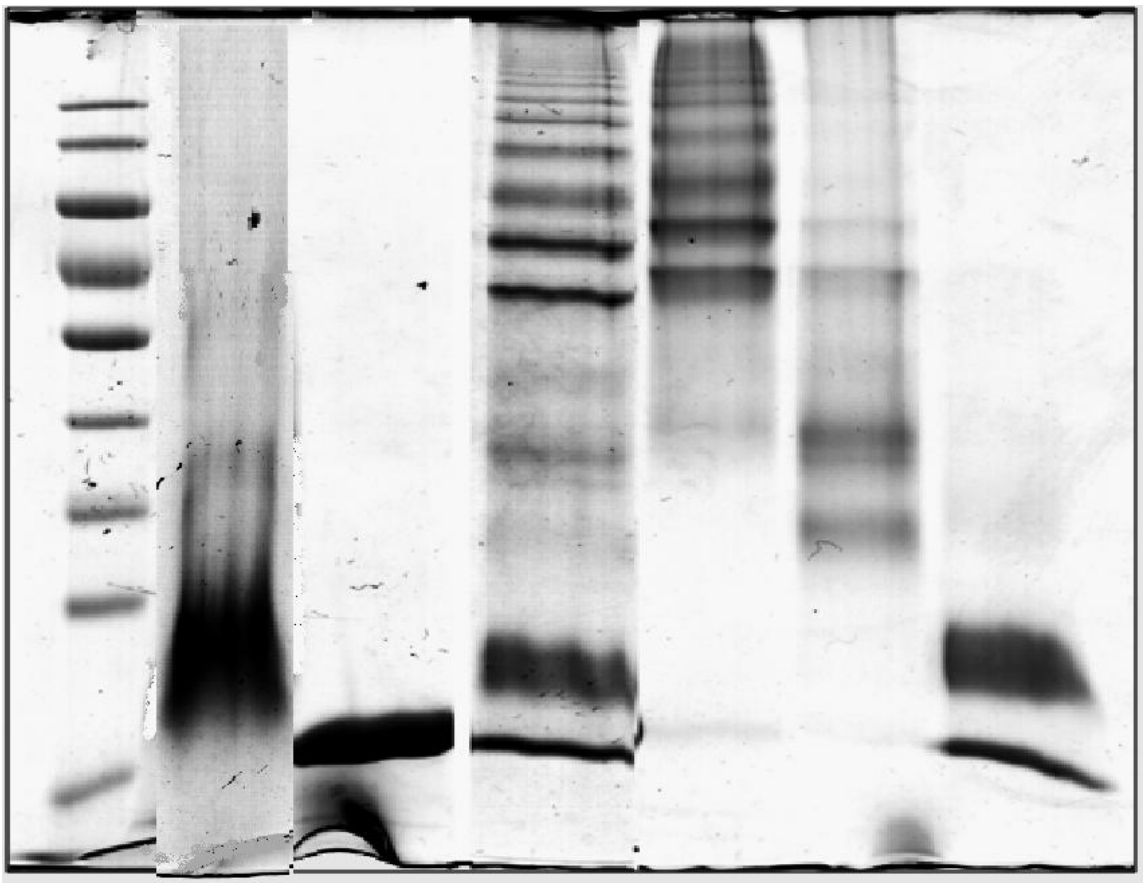


Figure 11: Absorbance spectrums of the collected solutions belonging to each of the three

To receive a clear identification of the products, gel analysis again was the most suitable method. Combined with the determined protein amounts, an accurate statement about the success of the reaction is possible.

SDS-PAGE after purification

The gel electrophoresis was performed exactly as previously described, including the purification of all samples containing GuHCl.



Gel B

1. PageRuler standard
2. PAMAM-DBCO (dialysed)
3. Aha-Ec1
4. Conjugate before purification

After ÄKTA purification:

5. Fractions of peak 1
6. Fractions of peak 2
7. Fractions of peak 3

Figure 12: Gel electrophoresis of Ec1, PAMAM-DBCO and the conjugates before and after ÄKTA purification. 12% SDS-polyacrylamide gel - 1nmol of each sample was applied , if not indicated otherwise

Figure 12 shows following results:
The gel displays clear and successfully separated products. All components detected is the reaction mixture (lane 4) were still separated and each found in one of the fractions. That result is evidence, that purification by ÄKTA under denaturing conditions is an appropriate method for isolating protein-PAMAM conjugates and separating unreacted educts.

Fraction 1 consisted of PAMAM-DBCO-Ec1 conjugates, but only those with a MW higher than 70 000 Da. According to previous calculations these conjugates made up about 53.7 % of the protein amount and so the biggest part.

Fraction 2, corresponding to peak 2 of the SEC chromatogram, represents a shoulder of peak 1, and was also made up of conjugates, but interestingly only those with a lower MW of 30 - 40 kDa, accounting for 10.9 %. Thus, the system was also able to separate conjugates according to their molecular weights, provided that, the fractions were kept apart, as demonstrated above.

Fraction 3 did not contain any conjugates, only unreacted PAMAM-(with and/or without DBCO) and unbound protein, basically the elements we intended to remove from the sample to avoid any confounding results of the evaluations. Regarding the spectrum of the ÄKTA purification, peak 3 was the only one that included an absorbance at 309 nm, the wavelength representing DBCO-NHS-ester. Together with previous results, this indicates that the absorption of DBCO is shifted or lost after successful reaction with an azido group.

Overall 23.3% of the total protein amount was associated with these unbound educts.
For comparison, almost 65 % resulted in PAMAM-DBCO-Ec1 conjugates, considering the fact, that about 12 % of the applied protein was not detectable after purification.

Concerning the upcoming siRNA complexation we used the conjugates with higher molecular weight (fraction 1), as they represented the biggest part. As described earlier, these conjugates include 3 or more protein molecules per PAMAM. However, they are covalently bound to the linker. So, the siRNA, which will be directly attached to the dendrimer should not be affected by the fact, that several different ratios of PAMAM to Ec1 were included.

5.3. siRNA complexation

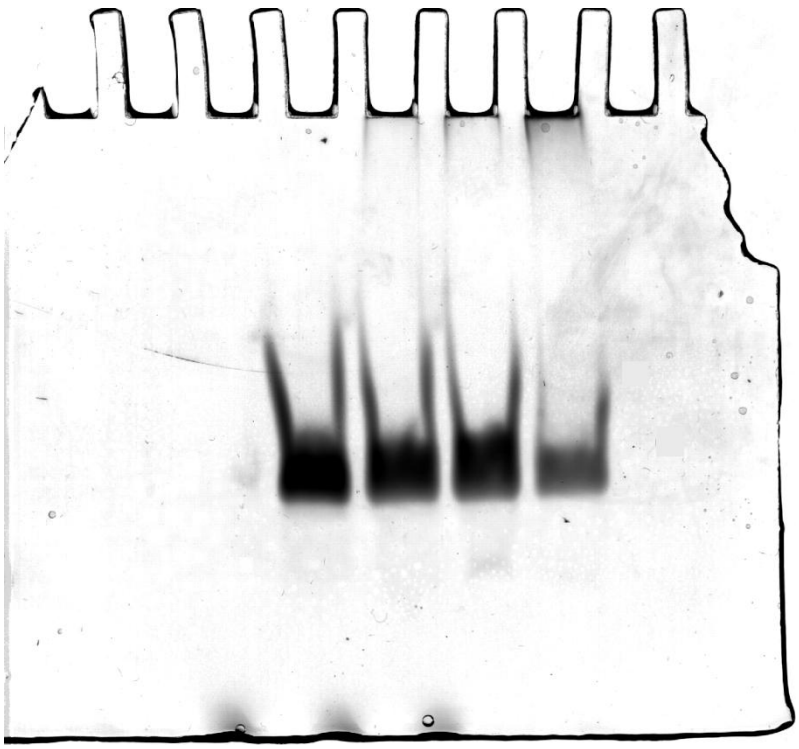
To attach siRNA to PAMAM-Ec1 conjugates, a neutral pH and native conditions are required. So there was still the problem, that the conjugates were solved in GuHCl, pH 8.0.
Several trials to rebuffer the samples into PBS buffer using dialysis or a Sephadex gel for purification, remained unsuccessful, either due to poor solubility or a low yield.

Anyway, after further attempts we found out, that adding siRNA to the conjugates in 3M GuHCl, without changing any conditions, improved the solubility of the entire mixture. Although the complexation would not take place yet, rebuffering the samples with coincident complexation of siRNA to the dendrimer became possible. So, after dialysing the samples against 1xPBS buffer, they remained in solution.

For this test an anti-Luciferase-based siRNA duplex was used. The main aim was to perform the complexation to PAMAM-Ec1 conjugates using variable N/P ratios and check the degree of conjugation under these conditions, using gel electrophoresis.

So solutions with three different ratios were prepared (N/P = 0.5; 1; 2) after calculating the exact amounts based on the number of positive amino charges of the conjugates deriving from PAMAM in relation to the number of negatively charged phosphate groups of the siRNA. That means, that increasing N/P ratios equate to a constant amount of siRNA, but rising amounts of the conjugates.
Furthermore, samples with the same N/P ratios were prepared for the cell culture tests, only using PAMAM G4 instead of PAMAM-Ec1 conjugates for comparison.

To have a reference for the extent of complexation, 0.5 nmol of siRNA were loaded into the first well.



Gel C

1. siRNA ctrl
2. siRNA + conjugate N/P 0.5
3. siRNA + conjugate N/P 1
4. siRNA + conjugate N/P 2

Figure 13: Complexation of siRNA with PAMAM-Ec1 conjugates. Each sample contained 0.5 nmol siRNA

As shown in figure 13, the first spot representing unbound siRNA is the most intensive one and could be used as a reference for densitometric determination. Only free siRNA could be detected and quantified, while conjugates of PAMAM-Ec1 and siRNA could not migrate and accumulated in the gel pocket.
The gel clearly proves that the capacity for complexation increased with rising N/P ratios. Starting at N/P 0.5 and moving towards N/P 2, the spots of free siRNA became less intense, while the bands of conjugates on the top became more visible.

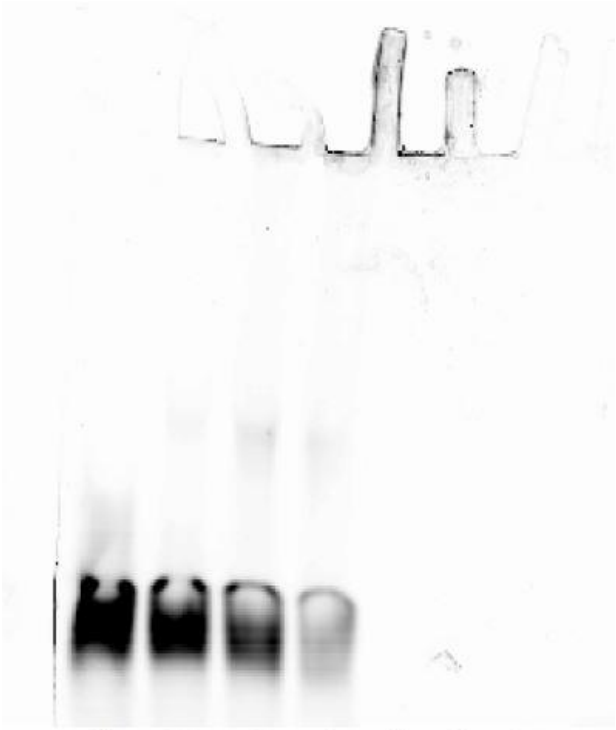
After scanning the gel with a densitometer, the intensity of each spot was measured and compared to free siRNA and the share that formed a complex was calculated.
Referring to the first spot as 100 % with the intensity of 5845.19, these were the results of the different samples:

N/P ratio	Intensity of spot	Free siRNA	Reacted share
0.5	5448.74	93.2 %	6.80 %
1	4893.47	83.7 %	16.3 %
2	2238.50	38.3 %	61.7 %

Table 7: siRNA binding capacity after complexation

The results indicate that in none of the applied N/P ratios, the complexation was complete. The highest complexation of 61.7 % was reached with N/P 2.

As a comparison this is a gel electrophoresis after the complexation of siRNA and PAMAM G4, instead of PAMAM-Ec1-conjugates, taken from a prior work [43]:



1 2 3 4 5 6 7

1. siRNA ctrl
2. siRNA + PAMAM N/P 0.1
3. siRNA + PAMAM N/P 0.25
4. siRNA + PAMAM N/P 0.5
5. siRNA + PAMAM N/P 1
6. siRNA + PAMAM N/P 2
7. PAMAM ctrl

Figure 14: Complexation with PAMAM G4. Each sample contained 0.5 nmol siRNA except for sample 7

Again, complexes of PAMAM and siRNA did not migrate and the visible spots represent free siRNA. Sample 1 (without PAMAM) shows the highest intensity. In contrast to the complexation with PAMAM-Ec1, it is obvious, that a complete complexation is more easily achieved with PAMAM G4.

Comparing single N/P ratios, the binding capacity was significantly higher in complexations without the DARPin. At N/P 1 and 2, no free siRNA was left. So the steric bulk of surface functionalization with protein limits complexation and a higher number of free surface amines is increases complexation.

5.4. Cell culture

5.4.1. Luciferase Assay

The main objective of the test was to check the cellular uptake and effect of PAMAM-Ec1 conjugates in EpCAM-positive (MDA-MB-468) and EpCAM-negative (HeLa) cells. Therefore, the cells were first transfected with luciferase plasmids, which enable the detection of luminescence signals. After successful delivery to the cytosol, anti-luciferase siRNA would lead to the reduction of the luminescence signal. The extent of this reduction is proportional to the degree of cellular uptake and consequently the gene silencing effect.

PAMAM generally is supposed to enable membrane passage, while the DARPin should allow specific targeting of cells expressing EpCAM. Therefore, the test was performed with conjugates containing AhaEc1 and simple PAMAM-siRNA complexes in the same N/P ratios.
By these means, it was possible to compare specific and unspecific uptake. For instance, by using different N/P ratios with increasing amounts of PAMAM and the involved interactions between positive surface charges of amines with negatively charged membranes, the role of unspecific uptake could be estimated. Furthermore, different amounts of the conjugates were tested for each ratio complying with 50 or 100 pmol of siRNA to check possible differences on the effect.

Unfortunately, the results of the assay were not fully reliable. In both, MDA-MB-468 and HeLa cells, the determined values did not show sufficient consistency, to be able to draw any conclusions regarding the different tested cases.
Table 8 and 9 show the average luminescence values as part of the signals detected in untreated cells.

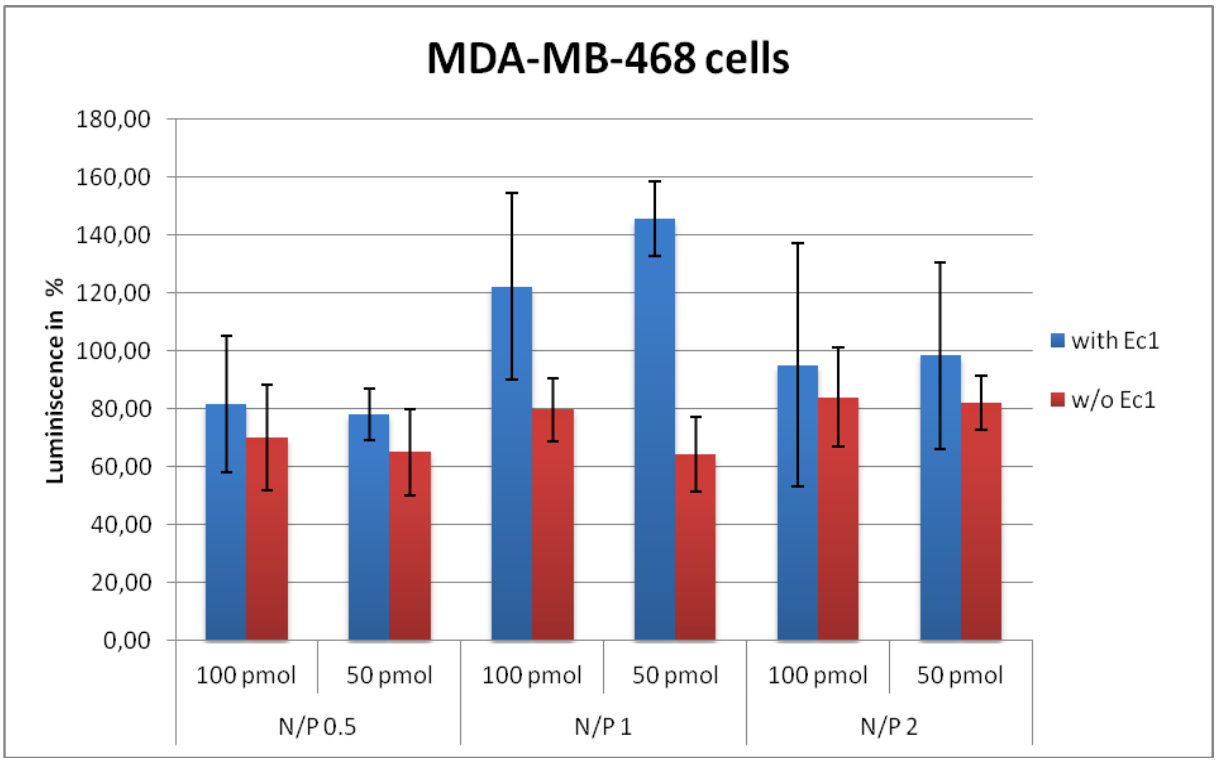


Figure 15: Results of MDA-MB-468 cells

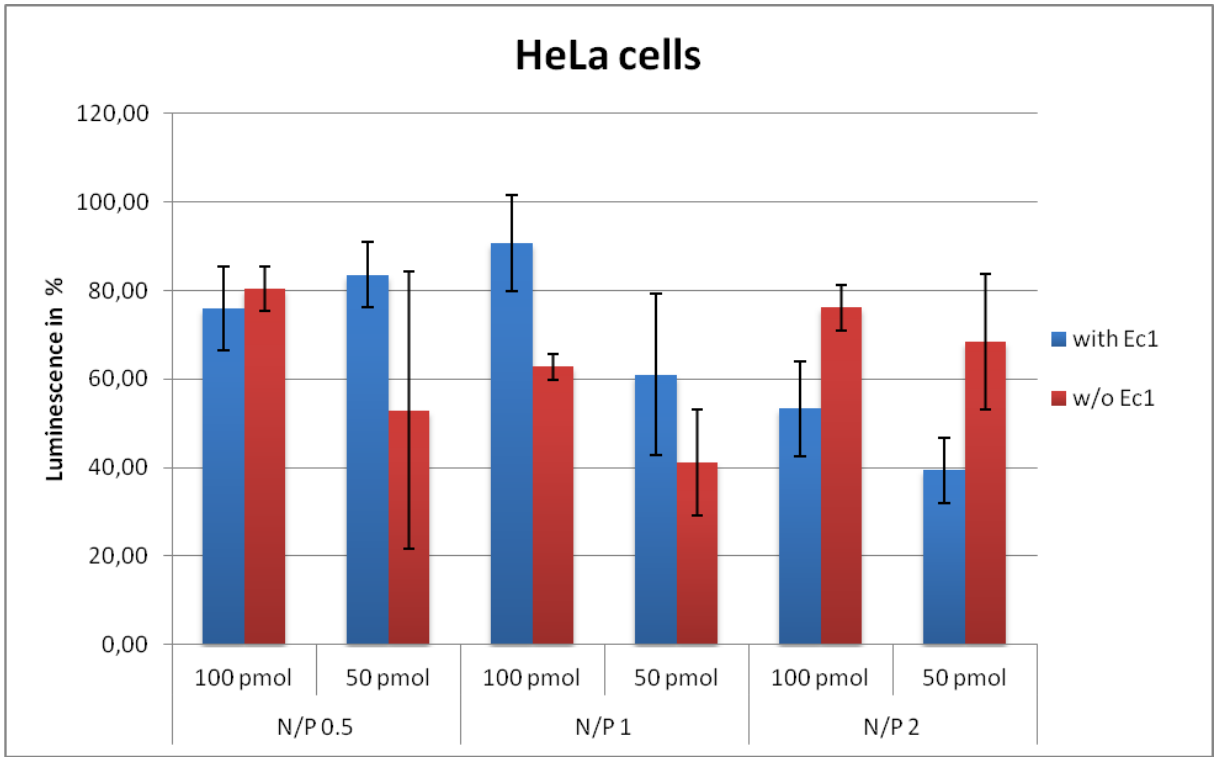


Figure 16: Results of HeLa cells

The results indicate that conjugates are generally less efficient in both cell lines than PAMAM. For EpCAM-positive MDA-MB-468 cells, a less pronounced uptake through receptor-mediated endocytosis compared to cationic charge mediated PAMAM transfection is expected. In EpCAM-negative HeLa cells, negligible uptake of the conjugates was expected; the luminescence down regulation is attributed to unspecific transfection caused by remaining positive surface charges of the conjugates. PAMAM would lead to efficient cellular uptake and luminescence down regulation regardless of EpCAM expression.

Although there are some indications of receptor-mediated uptake at N/P 0.5, the overall results are somewhat inconclusive and show high variations with no obvious correlation to concentrations or N/P ratios. This outcome is possibly due to variation in plasmid transfection efficiency.

5.4.2. Binding of fluorescent siRNA to EpCAM-positive and –negative cell lines

This test was carried out to check and quantify the delivery of PAMAM-Ec1-siRNA conjugates to MDA-MB-468 and HeLa cells by flow cytometry. All tested conjugates were produced using Alexa fluor 568 labeled siRNA. The assay is generally capable of indicating the binding capacity of conjugates.
To estimate the role of specific cellular uptake, conjugates of PAMAM and siRNA were also tested in the same three N/P ratios (0.5, 1, 2).

Comparing MDA-MB-468 and HeLa cells, it was obvious, that compounds containing AhaEc1 were binding significantly higher to EpCAM-positive MDA-MB-468 cells, as shown in the histograms (Figure 17 and 19). However, there was no clear difference between PAMAM-siRNA conjugates regarding the two cell types. Thus, a unique cell-specific recognition provided by the DARPin, is definitely present. Also, there was a direct correlation between the measured values and increasing N/P ratios.

In HeLa cells the binding of PAMAM-siRNA complexes was higher than conjugates with AhaEc1 in all ratios. The same applies to MDA-MB-468 cells, where values of conjugates without protein were significantly higher.

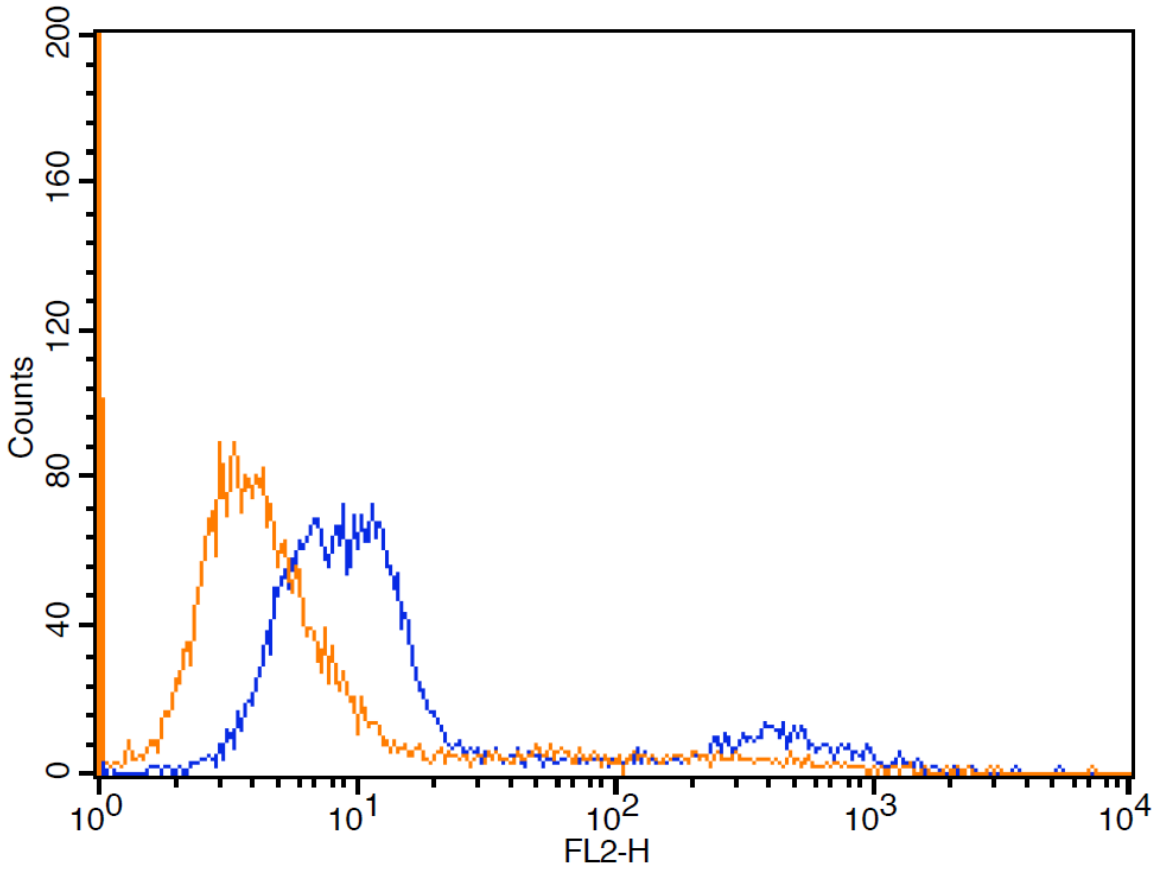


Figure 17: : Histogram of PAMAM-Ec1-siRNA conjugates with N/P 2
(blue= MDA-cells; orange= HeLa -cells)

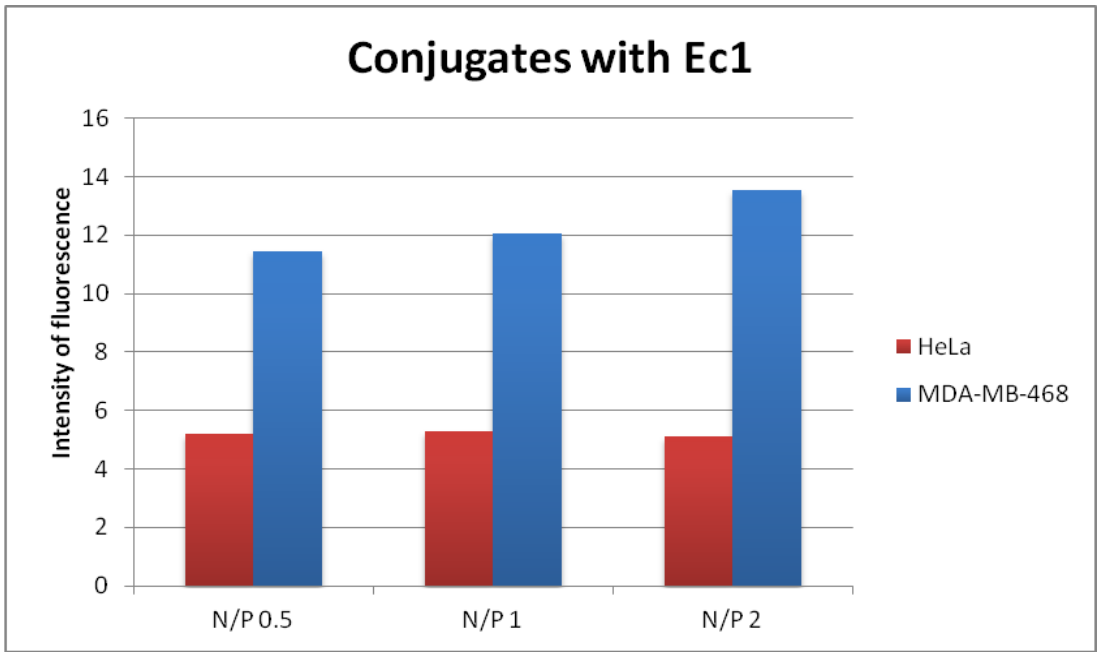


Figure 18: Fluorescence of PAMAM-Ec1-siRNA conjugates

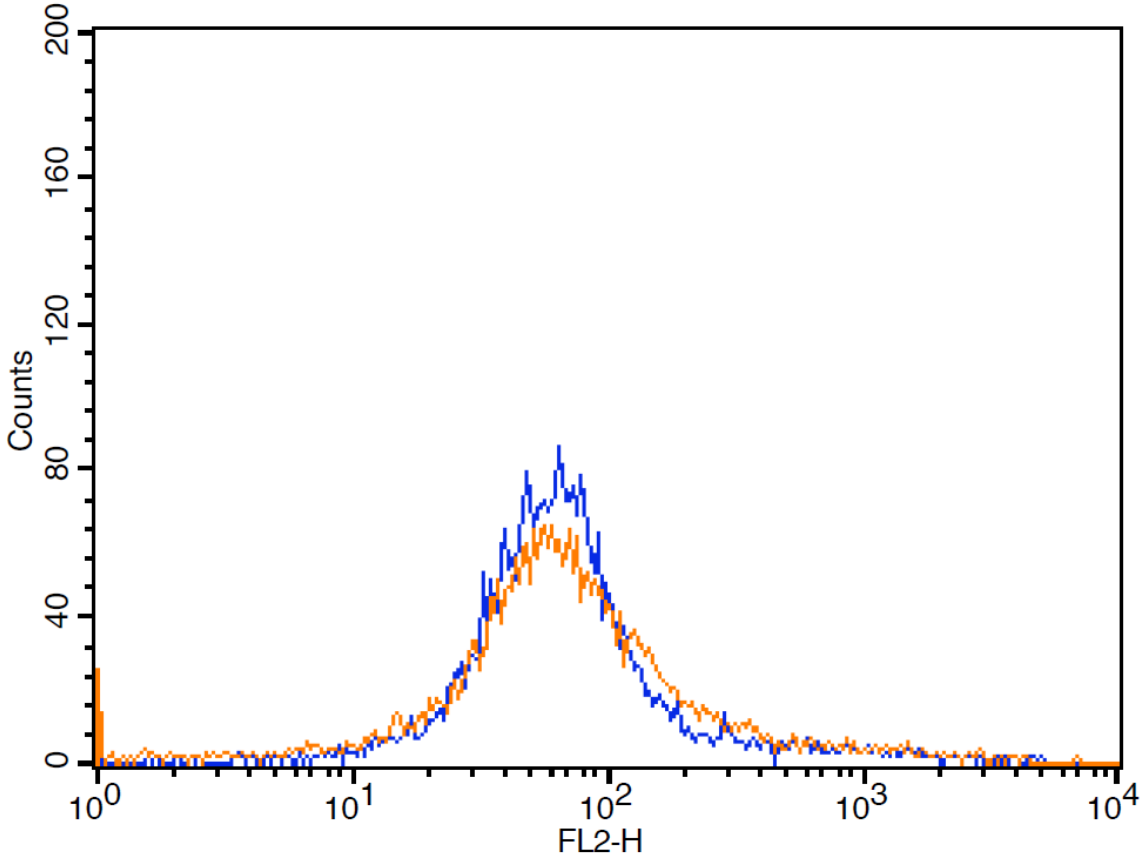


Figure 19: Histogram of PAMAM and siRNA conjugates without Ec1 with N/P 2
(blue= MDA-cells; orange= HeLa -cells)

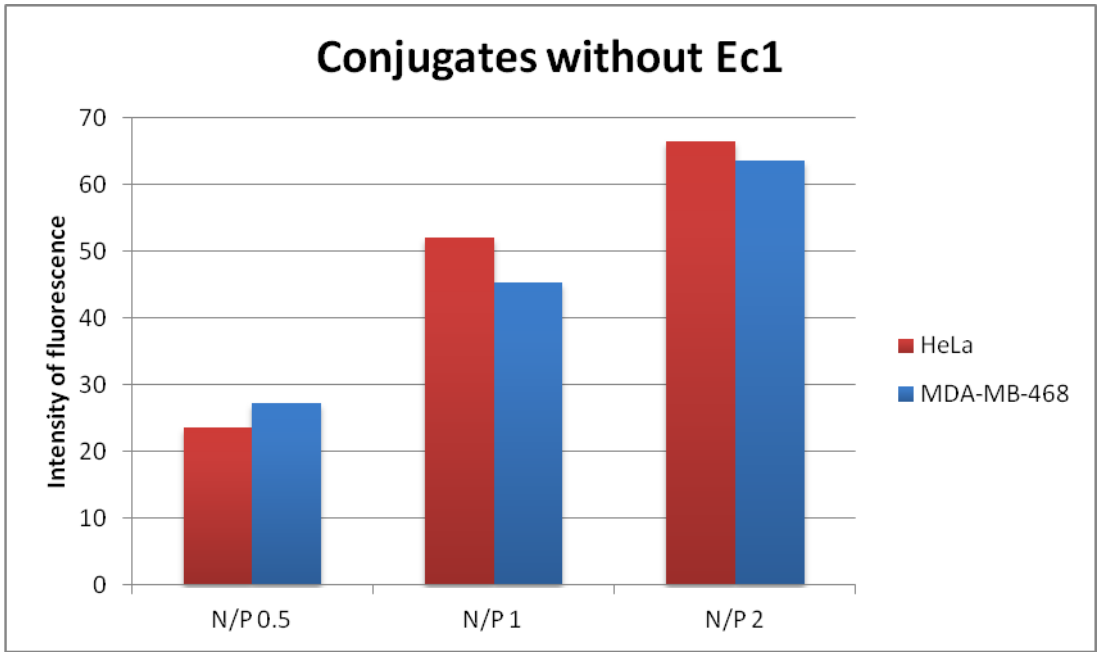


Figure 20: Fluorescence of PAMAM and siRNA conjugates without Ec1

A possible way to explain this outcome, is, that in EpCAM-positive cells, the binding and uptake occurs mainly via receptor recognition and binding. So the amount of conjugates, that can be delivered into the cells is limited in dependence on receptor saturation. On the other hand, when the DARPin is not present, the compounds, are able to bypass cell membranes by the effect of PAMAM, which is a much more efficient process than receptor-mediated uptake.

Thus it can be concluded that PAMAM results in efficient cellular uptake, but lacks any cell-type specificity. The Ec1-conjugates on the other hand bind significantly higher to receptor-positive cells, but the binding capacity and the cellular uptake is less efficient than with PAMAM. At the end, a certain amount of siRNA needs to be delivered to the cytosol, and cell-type specificity would enable targeting to additional tissues or achieve tumor-specificity.

Conclusion

During this work, chemical, biochemical and pharmacological methods were applied to produce and optimize delivery systems for siRNA therapeutics including PAMAM and the DARPin AhaEc1 for targeting EpCAM expressing tumor cells.
First, conjugations of PAMAM and DBCO linker were synthesized, followed by attachment of the DARPin Ec1 by click chemistry.
The obtained conjugations were checked by gel electrophoresis and spectrophotometric determinations. The reactions were successfully optimized and guanidine hydrocloride was identified as the ideal buffer.

The evaluation of different methods showed that size exclusion chromatography was providing the best properties for efficient removal of unreacted educts. siRNA was successfully complexed to purifed PAMAM-Ec1 conjugates.

Cell-specific binding of fluorescent siRNA complexed to conjugates was determined by flow cytometry. DARPin conjugates specifically bind to EpCAM-positive MDA-MB-468 cells in a much higher extent than to EpCAM-negative HeLa cells. PAMAM-mediated delivery was proven to be more efficient, but unspecific.

Finally, the work clearly fulfilled the anticipated aims of the biochemical part by optimizing the conditions and yields for different reactions. Regarding the cell culture tests, FACS also achieved valuable results, while the luminescence Reporter Assay didn't seem to be the ideal method for testing the produced conjugates.

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