In vivo evaluation of polymeric siRNA nanoformulations for tumoral gene silencing

von Johannes Schmaus

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität
München

In vivo evaluation of polymeric siRNA nanoformulations for tumoral gene silencing

von Johannes Schmaus

aus Oettingen in Bayern

München 2020

Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Lehrstuhl für Molekulare Tierzucht und Biotechnologie

Arbeit angefertigt unter der Leitung von: Univ.-Prof. Dr. Eckhard Wolf

Angefertigt am: Lehrstuhl für Pharmazeutische Biotechnologie, Fakultät für Chemie und Pharmazie der Ludwig-Maximilians-Universität München

Mentor: Univ.-Prof. Dr. Ernst Wagner

Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.
Berichterstatter:	Univ.-Prof. Dr. Eckhard Wolf
Korreferent/en:	Univ.-Prof. Dr. Johannes Hirschberger

Tag der Promotion: 25. Juli 2020

For those who left us way too early and those who will join us in the future.

TABLE OF CONTENTS

ABBREVIATIONS

°C	degree Celsius
µL	microliter
µm	micrometer
µg	microgram
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BSA	bovine serum albumin
BUN	blood urea nitrogen
cDNA	complementary DNA
CLSM	confocal laser scanning microscopy
Cy3	cyanine 3
Cy7	cyanine 7
ΔCT	delta cycle threshold
DBCO	dibenzocyclooctyne
dl	deciliter
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
e.g.	exempli gratia (for example)
EG5	eglin 5, kinesin spindle protein
EDTA	ethylenediamine tetraacedic acid
EGFR	epidermal growth factor receptor
EPR effect	enhanced permeability and retention effect
FCS	fetal calf serum
FolA	folic acid
FR	folate receptor
G	gauge
g	gravity
GADPH	glyceraldehyde 3-phosphate dehydrogenase
h	hour(s)
HA	hyaluronic acid
HBG	HEPES buffered glucose

i.v.	intravenous
IVIS®	*in vivo* imaging system
l	liter
LPEI	linear polyethylenimine
mg	milligram
min	minute(s)
ml	milliliter
mRNA	messenger RNA
N/P	polymer nitrogen to nucleic acid phosphate ratio
NIR	near infrared
nm	nanometer
U	units
OleA	oleic acid
PBS	phosphate buffered saline
pDNA	plasmid DNA
PFA	paraformaldehyde
PEG	polyethylene glycol
qRT-PCR	reverse transcription polymerase chain reaction
RFC	reduced folate carrier
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNase	ribonuclease
s.c.	subcutaneous
S.E.M.	standard error of the mean
siRNA	small interfering RNA
Stp	succinoyl-tetraethylene pentamine
TfR	transferrin receptor
UPL	universal probe library
wt	wildtype

I. INTRODUCTION

Cancer is the first or second leading cause for death in many countries (e.g. Germany) with almost 10 million deaths versus an incidence of about 18 million worldwide in the year 2018 [1]. In Germany the number of cancer diagnoses has risen from close to 1.5 up to over 1.6 million from 2006 to 2016 [2], which equates to a rise of over 10% within 10 years, although population was constant. Males diagnosed with cancer most often exhibit affection of the lung, while female patients most commonly suffer from breast cancer. Independent of sex, cancer diagnosis rates are increasing with age for most cancer types [1]. Other risk factors for coming down with cancer are inter alia alcohol and tobacco use, overweight, missing physical activity and inadequate nutrition [3]. These facts and numbers illustrate the importance of effective treatment options.

Tumor cells emerge from normal healthy cells that mutate for diverse reasons. Subsequently these alterations can lead to malignant tumor development. Responsible for this is the genetic makeup next to a variety of external biological, chemical and physical causes and of course the aforementioned ageing [3].

Once a patient is suffering from cancer, the sooner he is diagnosed the better are his future prospects concerning survival, under all circumstances and for the plural of cancer types. For this reasons, regular screenings have been established for certain cancers and can help to diagnose at an early timepoint. To treat tumors effectively an accurate tumor diagnosis is vital to initiate treatment. Common established treatment options, including surgery, radio- and chemotherapy, have their limitations. Metastasis e.g. restricts the potential of surgery and radiation because removing respectively erasing the original tumor is not sufficient in these cases. Furthermore, tumor cells can become resistant to conventional chemotherapeutic agents, which means they negate and disable the mode of action of these drugs and therefore their therapeutic potential [4].

Considering these problems of common treatment regime, new innovative approaches like gene therapy that depend on the reliable transport (e.g. by

non-viral vectors) into the target cell [5], are urgently needed. Hence, this thesis focuses on the evaluation of sequence-defined polymeric carriers for effective and tumor selective delivery of small interfering RNA (siRNA) using hyaluronic acid (HA) as a shielding and targeting agent.

1. Nucleic acid treatment via siRNA

Genomic analysis enabled the identification of multiple genes that are involved in tumor formation and spread [6]. This leads to the conclusion that these genes constitute possible targets for cancer therapy [7]. Possible candidates are nucleic acids containing drugs that can modify the expression of tumor related genes [8]. The group encompasses among other things messenger RNA (mRNA) and plasmid DNA (pDNA) [9] that lead to an increased gene expression, whereas siRNA (double-stranded) and antisense oligonucleotides (single-stranded) achieve the opposite by impeding functional mRNA [10, 11].

siRNA is a non-coding RNA that interferes directly with gene expression. The resulting specific knockdown of target genes predestines it for therapy of different diseases (e.g. cancer) [12-15]. Consisting of two strands, each comprising 21 to 23 nucleotides, siRNA is cleaved after successful delivery into the cytoplasm. The sense strand is disassembled, while the antisense strand is incorporated into the RNA induced silencing complex (RISC), which facilitates subsequent target mRNA identification followed by RNase initiated mRNA cleavage (illustrated in **Figure 1**). The mRNA inactivation therefore inhibits formation of disease-associated proteins [10, 12, 16, 17].

How promising this approach can be, demonstrates the drug approval of Patisiran (Onpattro^{TM}) in 2018 for the US market [18]. It is a liposomal siRNA formulation directed against hereditary transthyretin-mediated amyloidosis (hATTR) and the first approved siRNA-based drug. Another promising candidate is Givosiran (Givlaari^{TM}), an siRNA-based drug that combats acute intermittent porphyria (AIP) by interfering with aminolevulinic acid (ALA) synthesis, which plays a role in AIP pathogenesis, was approved in November 2019 for medical use [19, 20].

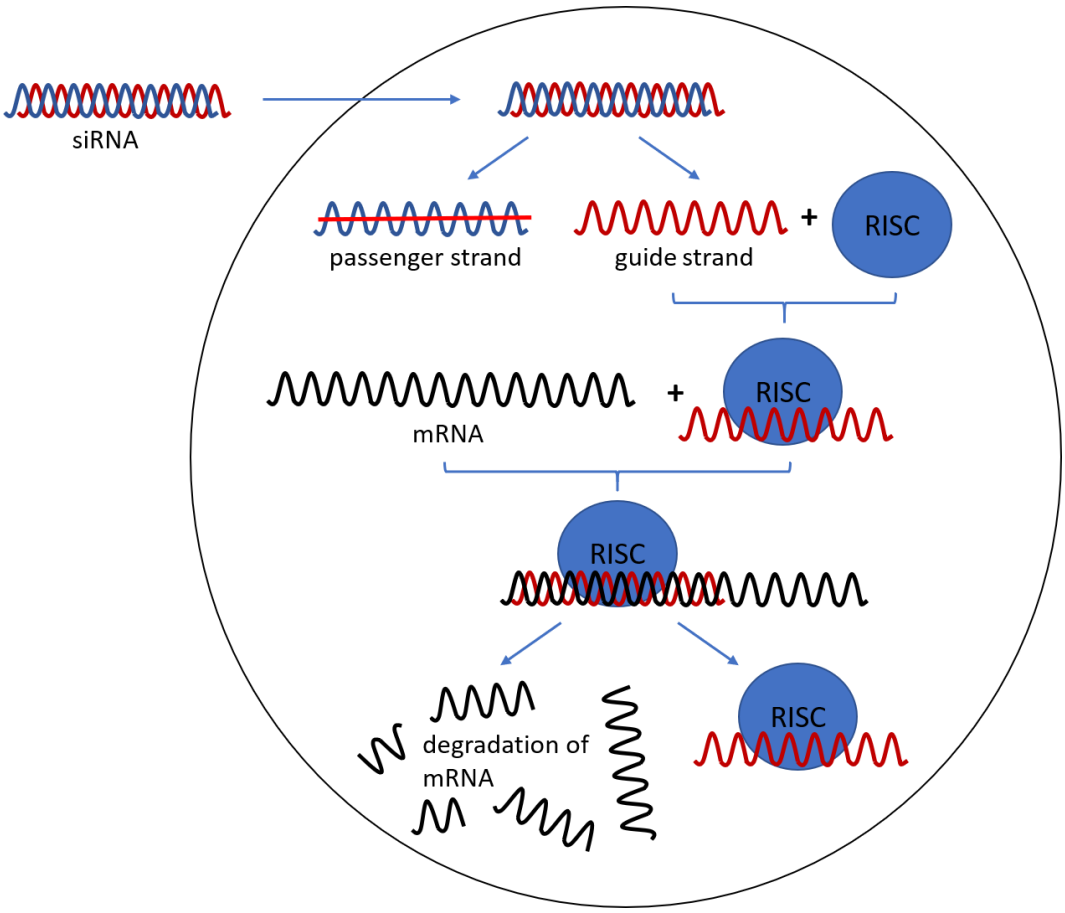


Figure 1: Gene silencing effect of siRNA. After endocytosis and release into the cytosol, the two strands of siRNA are separated. While the sense (=passenger) strand is dismantled, the antisense (= guide) strand undergoes incorporation into the RISC complex. This leads to the identification and subsequent RNase-initiated degradation of the corresponding target mRNA and results in a reduction of disease-associated gene expression [21].

1.1. Hurdles

Concerning tumor targeting, a recent study states that less than 1% of injected nanoparticle formulation ultimately reaches tumor tissue [22]. SiRNa delivery in particular is facing hurdles, like insufficient tolerability and circulation time [23] that have to be solved for efficient and sustainable disease treatment, especially for non-liver diseases. Pharmacokinetic characteristics of pure siRNA are inadequate and therefore present half-lives limited to minutes after systemic application [24, 25]. An optimal transport system for instable siRNA is therefore crucial and has to overcome manifold obstacles like nuclease-triggered degradation [26] and undesired agglomeration with blood components, both negatively affecting time of circulation [23]. Additionally reactions of the immune system have to be evaded by these carriers [12, 27, 28]. Since a particle size below 10 nm induces unwanted renal filtration, introducing carrier systems can help to

increase the nanoparticle size to a desired limit of around 200 nm [29]. If the created particles are stable enough to reach tumor tissue via bloodstream, they can passively extravasate due to tumor vessels being more permeable, because of gaps between endothelial cells, compared to healthy tissues [30-32]. Besides, a recent study suggests that entering tumor tissue might be an active process, e.g. binding to endothelial cells followed by trans-endothelial delivery [33]. Once the formulated siRNA has accessed tumor tissue, the endosomal uptake into tumor cells is possible (illustrated in **Figure 2**). The subsequent endosomal escape that can be affected by correctly chosen carriers, finally enables free siRNA to cleave target mRNA (illustrated in **Figure 1**).

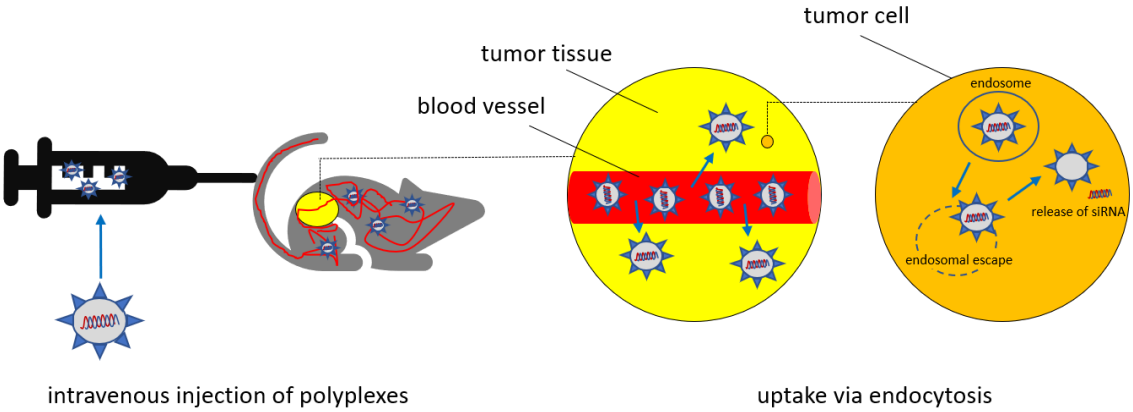


Figure 2: Delivery of siRNA into tumor cells. After intravenous injection of siRNA polyplexes into tail vein and transport in the blood (**red**), a fraction of the particles enters tumor tissue (**yellow**). Formulations can pass the cell wall and enter the tumor cell (**orange**) by endocytosis. The formed endosome releases siRNA into the cytosol after pH-triggered endosomal escape [21].

1.2. Carrier systems

The aforementioned barriers can be bypassed with the usage of carrier systems with definite characteristics.

Basically, one can choose between viral and non-viral vectors, which are the two main groups when looking for potent transporters. While viral vectors can transfect target cells effectively, what can be explained with their viral origin, and mostly replication as well as cytotoxicity pose no threat, their major disadvantages are immunogenic reactions and genomic mutations [34, 35]. In addition, production of sufficient amounts constitutes

an issue [36].

An encouraging option besides viral vectors to deliver siRNA, are non-viral vectors like cationic polymers and liposomes [37]. In combination with anionic nucleic acids like siRNA stable nanoparticles, so-called polyplexes [38], can form. An important cationic representative is linear polyethylenimine (LPEI) [39], though it has major deficits regarding reproduceable and lasting stability [40], immune response and biodegradability [41].

Therefore, this work evaluates carriers with positive charge that are created via solid-phase assisted synthesis. To this day our group developed around 1400 sequence-defined small peptide-like polymers, so-called oligomers. These small polycations dispose over different moieties concerning structure and function, like the proton-sponge that induces swelling by an osmotic gradient and proximate disruption of the endosome [42, 43], to facilitate gene delivery. Their reduced size and positive charge make them superior compared to other polymers, especially concerning cell toxicity.

Exact sequence-defined oligomers were created for the first time in 2006 [44] and subsequently advanced by members of our work group by increasing the number of building blocks [45] respectively further structures like a polyethylene glycol chain combined with terminal folic acid as targeting ligand [46].

In the described polymer **1214** (illustrated in **Figure 3**), the cationic backbone consists of the artificial amino acid succinoyl tetraethylene pentaamine (Stp). This polymer building block is positively charged, due to its partly protonation status at neutral pH, which enables polyplex formation when combined with negatively charged siRNA [47].

Additional structures are added for positive influence. Amino acids have varying effects: tyrosines and cysteines enhance stability by their hydrophobic character respectively by forming disulfide bridges [48, 49], while histidines support endosomal escape by their proton sponge effect [43] and by increasing the endosomal buffering capacity [50]. Azido-lysines were introduced to allow post-functionalization with dibenzocyclooctyne (DBCO) click agents e.g. hyaluronic acid (HA) via strain-promoted alkyne-

azide cycloaddition (SPAAC) [51, 52]. This azide function enables introduction of shielding as well as targeting ligands onto the surface after polyplex formation by smoothly adding DBCO derivates (illustrated in **Figure 4**).

Like tyrosine, the fatty acid, oleic acid, has a comparably positive effect on polyplex stability, which is important in particular for siRNA delivery [53]. In addition, it improves the lysis of the lipid membrane of the endosome during the process of endosomal escape [54].

The topology of the polymer also impacts the way siRNA can be incorporated into the resulting polyplex. To deliver siRNA, T-shaped oligomers, that normally consist of two hydrophobic fatty acids bound to the positively charged Stp-backbone, demonstrated high aptness [55].

After polyplex formation the polymer surface can be equipped with shielding as well as targeting ligands that limit unwanted interactions and enable cell specific uptake. Besides from nucleic acid delivery, drug delivery in general, even in parallel [56], might be an option, due to optimization of structure and function that encompass optimal size and active and passive targeting.

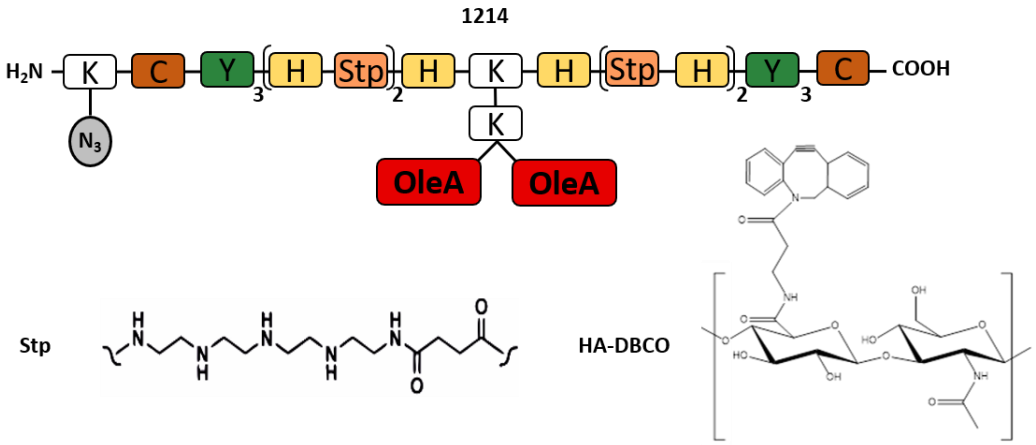


Figure 3: Simplified depiction of compounds for polyplex formation. **Top**: T-shaped oligomer (ID: **1214**) containing two oleic acids (**OleA**). Further elements of the oligomer: C: cysteine, H: histidine, Y: tyrosine, K: lysine, Stp: succinoyl-tetraethylene-pentamine, N_3: azide function. **Bottom**: chemical structure of **Stp** on the left respectively of shielding and targeting structure **HA**-DBCO on the right.

1.3. Shielding and targeting of polyplexes

To circumvent unwanted interactions with blood components, provoked by

the polymers cationic charge [57], and extend vascular circulation [58], effective particle shielding is essential, as it can further enhance passive targeting and combined with right particle size of 10 to 200 nm [59], ultimately boost the tumor specific enhanced permeability and retention (EPR) effect [60, 61]. Finally, incorporated ligands, e.g. directed against certain receptors, enable active targeting for tumor cell specific internalization [62].

Adding passive shielding ligands to polyplexes can hamper renal filtration as well as undesired interactions within the bloodstream by masking the positive charge and guaranteeing ideal particle size [63, 64]. Also, nuclease-initiated degradation processes are limited [65] and therefore enhance EPR effect by prolonged blood circulation time. Beneficial for increased tumor accumulation, accompanied by EPR effect, is the naturally enhanced permeability of tumor vessels due to gap junctions and pores between endothelial cells, which enables extravasation of the polyplexes [30-32]. In addition to increased vascularization of tumor tissue, lymphatic drainage is inadequate [66], increasing the amount of retained nanoparticles and minimizing particle removal. Though, a recent study suggesting that entering tumor tissue might be an active process, e.g. by binding to endothelial cells followed by trans-endothelial delivery and other mechanisms considered worthy of scientific study, has to be mentioned [33].

The aforementioned goals can be meet by the most common shielding agent polyethylene glycol (PEG) [67], although negative side effects like immunogenic reaction, e.g. production of antibodies directed against PEG, are described [68]. Because of these adverse immune reactions, scientists are looking for promising alternatives to PEGylation without the immunogenic disadvantages.

Referring to Whitesides' rules, ideal shielding candidates should contain polar functional groups with hydrogen bond acceptor characteristics, while hydrogen bond donor groups or net charge are absent [69]. Alternatives like repeats of proline-alanine-serine (PAS) [70, 71], oligosaccharides [72], the artificial polymer hydroxyethyl starch (HES) [73] fail to meet all the Whiteside criteria. Although polysarcosine (Psar), a polypeptoid, covers all of the aforementioned requirement and its protein resistance was validated

on different materials [74-76], still gene silencing was not achieved, while biodistribution revealed prolonged circulation time [77].

Another possible shielding candidate is the natural polysaccharide hyaluronic acid (HA), which can be found in the extracellular matrix and synovia of human body. Its shielding function results from its negative charge, which can reduce the cationic surface charge of the polymeric carriers and therefore limit nonspecific interactions with anionic blood components [78-80]. First studies give evidence of improved internalization and transfection with simultaneous low cytotoxicity *in vitro* [81] and *in vivo* [82]. Also, the formation of particles with an ideal maximum size of 200 nm is possible [83]. To sum that up, HA represents a promising shielding agent for *in vivo* evaluation. In addition, in contrast to the other mentioned agents, HA can act as receptor-ligand for cell uptake by endocytosis (see below).

Though, currently the relationship between surface charge and therapy efficacy is only partly understood [84, 85]. Like previously described, polyplexes with negative or positive charge are prone to enhancing blood circulation time and hence tumor accumulation, due to reduced interaction with blood components. On the other hand, one has to note the influence of angiogenic tumoral endothelial cells that line the interior of vascular walls, and ultimately play an important role in intratumoral transport followed by possible gene silencing. The positive effect on binding and internalization of positively charged liposomes into endothelial cells [85] and therapeutic superiority, regarding tumor suppression, of cationic towards anionic or neutral particles, carrying a chemotherapeutic, could be demonstrated [84]. For that reason, the investigation of differently charged polyplexes, entailed by varying HA concentration, regarding their attraction to angiogenic endothelial cells in tumor tissue, is part of this work.

Next to passive targeting via shielding agents in order to protect the surface [86] and enhance the EPR effect [61], active targeting by insertion of adequate ligands can be utilized to reach tumor tissue. Carriers have to fulfill two main goals: in the first place the siRNA polyplexes have to make their way to tumor tissue after systemic application, while maintaining their function [87], and in the second place be able to actively enter tumor cells with subsequent liberation of siRNA into the cytoplasm [66]. Specifically for

the second part, introduced active targeting ligands are widely used [88] and directed against fitting receptors. One advantage in tumor targeting is the overexpression of multiple receptors in tumor cells [89-91] that can represent possible objectives for suitable active targeting ligands.

The folate receptor (FR) [92-95], epidermal growth factor receptor (EGFR) [96, 97] or transferrin receptor (TfR) [98-100] are frequently targeted, because of their presence in a multitude of cancer types. A wide range of targeting ligands like peptides [101], proteins comprising antibodies [102], aptamers or glycoproteins [103] is available [104].

All three aforementioned receptors were target of previous studies in our lab. Folic acid (FolA) and methotrexate (MTX) with its similar chemical structure can internalize tumor cells via FR specific uptake or reduced folate carriers (RFC) [105] and were successfully utilized as ligands [106-111]. Transferrin containing oligomers were capable of internalization, endosomal escape and successful subsequent transfection for gene delivery [112, 113]. EGFR that is typical for solid tumors [114], is target for many antitumoral drugs, especially directed against lung cancer [115, 116]. The peptide GE11 exhibits one possible targeting ligand directed against EGFR for tumor cell specific oligomer delivery [117, 118], while avoiding mitogenic side effects [119].

In this thesis we focused on the natural polymer hyaluronic acid (HA) as a combined shielding and targeting agent as designed by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU)*. After siRNA incorporation, the azido-modified polyplex surface is functionalized with HA coupled to dibenzocyclooctyne (DBCO) by copper-free click reaction with the azido functionality (illustrated in **Figure 4**) [51]. HA can enter the cell via receptor-mediated endocytosis, employing its primary receptor, the transmembrane glycoprotein CD44 [120]. CD44 also represents an biomarker for cancer stem cells (CSC) that have multiple effects on tumor development [121], demonstrating the influence it can have by tumor targeting [122]. Furthermore, CD44 overexpression is reported for different cancer types comprising lung [123], pancreas [124] and breast tumors [125] compared to healthy tissue. Additionally, HA itself is biocompatible, provokes no immune response, which are in combination with its hydrophilic character, valuable

properties [126]. Several clinical trials focusing on CD44 as target structure [127-129], highlight the potential of HA as its main ligand.

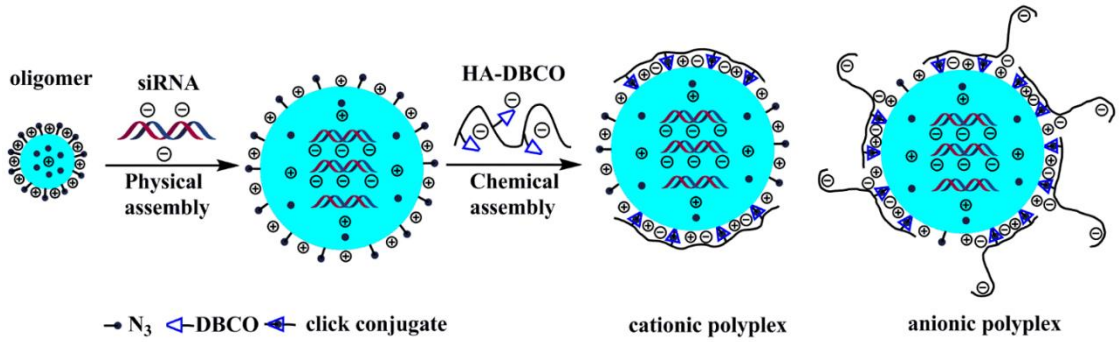


Figure 4: Simplified depiction of the polyplex creation. HA shielded and targeted polyplexes containing siRNA are formed via click-chemistry, which is enabled by the azide function and HA modified with DBCO. Before this surface functionalization, the cationic backbone can bind negatively charged siRNA. Depending on the amount of contained anionic HA, cationic or anionic polyplexes are created. The figure is provided by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU*).

2. Aims of the thesis

The thesis aimed at evaluation of the *in vivo* characteristics of siRNA nanoparticles developed for specific delivery into tumor tissue in a human tumor xenograft mouse model.

For that purpose, nanoparticles formulated with hyaluronic acid as shielding and targeting ligand of varying charge, depending on the amount of HA, were first to be tested in a human HUH7 hepatocellular carcinoma mouse model for their systemic biodistribution pattern, especially concerning tumor delivery.

In addition, efficacy of EG5 target gene silencing after twofold intravenous application of the different nanoparticles had to be evaluated (in collaboration with Jie Luo, *PhD student at Pharmaceutical Biotechnology, LMU*).

Furthermore, *in vivo* cytotoxicity of the different siRNA nanoparticles was to be determined via analysis of blood plasma for liver (alanine aminotransferase and aspartate aminotransferase) and renal parameters (blood urea nitrogen and creatinine).

Moreover, histology including immunostaining and 3D-reconstruction was to be performed (in collaboration with Mochen Cui, *PhD student at Center for Neuropathology and Prion Research, LMU*) to gain deeper understanding of the tumoral distribution pattern.

II. MICE, MATERIALS AND METHODS

1. Mice

1.1. Mouse strain

Female Rj: NMRI-Foxn1^{nu}/Foxn1^{nu} mice were purchased from Janvier Labs (Le Genest-St-Isle, France). As a result of a mutation in the Foxn1^{nu} gene, this outbred mouse strain lacks a thymus and therefore has no mature T-lymphocytes, whereas B-lymphocytes and other cells of the innate immune system are present and functionally working. These mice are nude, due to an impaired keratinization of hair follicle and epidermis. They are therefore an ideal model for bioimaging experiments and because of their incomplete immune system perfect for the usage in xenograft tumor models.

1.2. Housing conditions

Mice were purchased at an age of 5 weeks and acclimated for 7 days before starting experiments. Animals were housed in isolated ventilated cages (IVC type II, Tecniplast) under specific pathogen-free conditions, which were controlled as described in 1.3. A 12 h day / night interval was maintained, temperature and air humidity were kept constant at 24 - 26°C, respectively 50 - 70% and checked daily. Light and sound intensity were set to 200 Lux, respectively 40 dB. The stocking density of each cages varied from 2 to 5 mice. Cages were furnished with dust-free bedding (ABEDD Vertriebs GmbH, Österreich) that was changed once per week, whereas sterilized food (ssniff Spezialdiäten, Soest, Germany) and water were available *ad libitum.* The cages were enriched with cottages, wooden tubes and nest building material. All parameters were maintained according to §11 of the German Animal Welfare Act [130].

1.3. Health monitoring

Every 12 weeks health monitoring of the animal facility was conducted. For this reason, sentinel mice were held in the same animal facility and weekly received bedding material and food from all animal cages that were part of

a running experiment. Every 3 months, a complete health analysis of two sentinel mice was done by an external laboratory (mfd Diagnostics GmbH, Wendelsheim, Germany).

2. Materials

2.1. Cell culture

Cell culture was done by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU).*

Material	**Source**
HUH7 - wildtype cells (human hepatocellular carcinoma cells)	JCRB Cell Bank (Osaka, Japan)
DMEM 1 g/l glucose medium	Invitrogen (Karlsruhe, Germany)
FCS (fetal calf serum)	Invitrogen (Karlsruhe, Germany)
PBS (phosphate buffered saline)	Biochrom (Berlin, Germany)
TE (trypsin EDTA) solution	Biochrom (Berlin, Germany)
L-alanyl-L-glutamine	Biochrom (Berlin, Germany)
Cell culture plates and flasks	TPP (Trasadingen, Switzerland)

2.2. *In vivo* experiments

The experiments including perfusion were performed in cooperation with Mochen Cui (*PhD student at Center for Neuropathology and Prion Research, LMU*)

Material	**Source**
Isoflurane CP®	CP-Pharma (Burgdorf, Germany)
Xylavet	CP-Pharma (Burgdorf, Germany)
Ketamine	WDT (Garbsen, Germany)
Bepanthen®	Bayer Vital GmbH (Leverkusen, Germany)
Syringes, needles	BD Medical (Heidelberg, Germany)
Multivette (EDTA-coated tubes)	Sarstedt (Nümbrecht, Germany)

HBG (HEPES buffered 5% glucose, pH 7.4)	HEPES: Biomol (Hamburg, Germany) glucose monohydrate: Merck (Darmstadt, Germany)

2.3. Histology

Immunostaining and 3D reconstruction were done by Cui Mochen (*PhD student at Center for Neuropathology and Prion Research, LMU*) and Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU).*

Material	**Source**
PBS (phosphate buffered saline)	Biochrom (Berlin, Germany)
PFA (paraformaldehyde)	Sigma-Aldrich (Munich, Germany)
DyLight 488 labeled L*ycopersicon esculentum* (tomato) lectin	Vector Laboratories (Burlingame, CA, USA)
BSA (bovine serum albumin)	VWR (Radnor, PA, USA)
mounting medium H-1400	Vector Laboratories (Burlingame, CA, USA)
confocal microscope Zeiss LSM780	Zeiss (Oberkochen, Germany)

2.4. Oligomer

The oligomer **1214** (illustrated in **Figure 5**) was synthesized Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU).*

ID	**Sequence**	**Topology**
1214	$K(N_3)$-C-Y_3-$(H\text{-}Stp)_2$-H-$K(K(OleA)_2)$-H-$(Stp\text{-}H)_2$-Y_3-C	T-shape

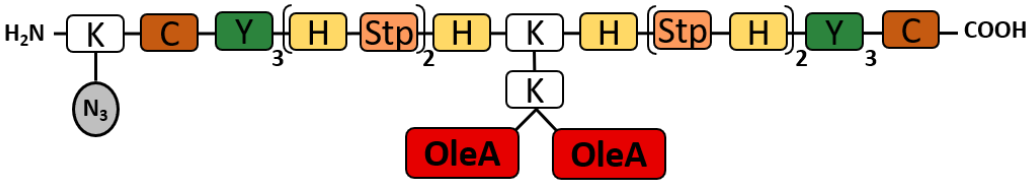


Figure 5: Illustration of polymer **1214**.

2.5. siRNAs

siRNA	Source
siCtrl AuGuAuuGGccuGuAuuAG dTsdT CuAAuAcAGGCcAAuAcAU dTsdT	Axolabs (Kulmbach, Germany)
siEG5 ucGAGAAucuAAAcuAAcu dTsdT AGUuAGUUuAGAUUCUCGA dTsdT	Axolabs (Kulmbach, Germany)
siAHA1-Cy7 GGAuGAAGuGGAGAuuAGu dTsdT (Cy7)(NHC_6)ACuAAUCUCcACUUcAUCC dTsdT	Axolabs (Kulmbach, Germany)
siAHA1-Cy3 ACuAAUCUCcACUUcAUCC dTsdT (Cy3)(NHC_6)GGAuGAAGuGGAGAuuAGu dTsdT	Axolabs (Kulmbach, Germany)

* Capital letters: standard RNA ribonucleotides (A: Adenylate, G: Guanylate, C: Cytidylate, U: Uridylate); small letters: 2'methoxy-RNA; dT: deoxy-thymidine; s: phosphorothioate linkage

Conjugates were provided by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU).*

2.6. Compounds

Hyaluronic acid (HA)	Lifecore Biomedical (Chaska, MN, USA)

The amount of DBCO on HA is described as molar equivalents (equiv.) related to the cationic oligomer (mol/mol).

2.7. Instruments

Name	Manufacturer
Caliper DIGI-Met	Preisser (Gammertingen, Germany)
IVIS Lumina	Caliper Life Science (Rüsselsheim, Germany)
Microtome Leica VT1000S	Leica (Wetzlar, Germany)
Confocal microscope Zeiss LSM780	Zeiss (Oberkochen, Germany)

2.8. Software

Name	Manufacturer
GraphPad Prism 5 software	GraphPad Software (San Diego, USA)
Living Image 3.2	Caliper Life Science (Rüsselsheim, Germany)
Imaris 9.0.1	Bitplane (Belfast, UK)

3. Methods

3.1. Cell culture

Human hepatocellular carcinoma cells (HUH7) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 g/L glucose, 4 mM stable glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cell line was cultured at 37 °C and 5 % CO2 in an incubator with a relative humidity of 95%.

3.2. *In vivo* experiments

HUH7 tumor cells were suspended in 150 µL PBS and injected subcutaneously into the left flank of 6-week-old mice using a 27G cannula. Inoculation was done under inhalation anesthesia (5% isoflurane in oxygen for induction and 2.5% for maintenance).

Animal weight was determined every second day until tumors became measurable and from then on daily. Tumor sizes were measured by caliper and calculated using the following formula: 0.5 × longest diameter × shortest diameter^2 [131]. Intravenous injections into the lateral tail veins were performed using a restrainer and prior to the injection tails were warmed in lukewarm water (~37°C) to depict the veins more clearly.

Euthanasia by cervical dislocation was performed under isoflurane narcosis when previously determined abort criteria occurred (for example a tumor diameter of 12 mm or a severely affected well-being).

All animal experiments were approved by the district government of Upper Bavaria (reference numbers: 55.2-2532.Vet_02-19-020 and 55.2-1-54-2532-233-13) in accordance with the guidelines of the German Animal

Welfare Act [130].

3.2.1. *In vivo* biodistribution studies

6-week-old mice were injected subcutaneously into the left flank with 5 × 10^6 HUH7 tumor cells. As soon as the tumors reached 500 mm³, animals were randomly divided into 7 groups (n=3). They received a single i.v. injection with 250 µL of **1214** with **0.04** up to **1.0** equiv. of HA-DBCO modification comprising 50 µg Cy7-labeled (50%) siRNA at N/P 12. Using a CCD camera NIR fluorescence bioimaging was conducted under inhalation anesthesia with 3% isoflurane in oxygen at different time points for 24 h. After the last timepoint mice were sacrificed and images of tumor and organs were obtained. For the image evaluation color bar scales were equalized and efficiency of fluorescence signals was analyzed using the Living Image software 3.2.

3.2.2. Clinical blood parameters

During the EG5 gene silencing studies blood was obtained from all mice by final heart puncture after euthanasia by cervical dislocation. The blood samples were gathered in EDTA-coated tubes and centrifuged at 3000 rpm for 7 minutes to isolate blood plasma. The supernatant was analyzed for liver (alanine aminotransferase and aspartate aminotransferase) and renal parameters (blood urea nitrogen and creatinine) in the *Clinic of Small Animal Medicine, Faculty of Veterinary Medicine, LMU*. Untreated mice injected with HBG served as reference.

3.2.3. *In vivo* gene silencing study

6-week-old mice were injected subcutaneously with 5 × 10^6 HUH7 cells and divided into 5 groups (n=5) when tumors reached a size of 500 mm³. Mice were injected i.v. with a volume of 250 µL of **1214** with **0.1** equiv. of HA-DBCO modification respectively **0.8** equiv. of HA-DBCO modification comprising 50 µg of siCtrl respectively siEG5 at N/P 12. Control animals received the solution buffer HBG. The previously described formulations were administered twice with a twenty-four hours interval. 24h after the second and last injection the mice were sacrificed. In parallel with euthanasia blood was obtained by heart puncture for the following clinical biochemistry (see 3.2.3).

Tumors were gathered to extract RNA by using Trifast (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. Gene silencing efficiency was evaluated by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU*). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure the EG5 mRNA level in tumor tissue. Total RNA was isolated followed by reverse transcription using qScript™ cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). Quantitative RT-PCR was performed in triplicates on a LightCycler 480 system (Roche, Mannheim, Germany) using UPL Probes (Roche, Mannheim, Germany) and Probes Master (Roche, Mannheim, Germany) with GADPH as housekeeping gene. The following probes and primer sequences were used: human GAPDH (ready-to-use in UPL, UPL Probe #45), and human EG5 (UPL Probe #53) (forward: CATCCAGGTGGTGGTGAGAT, reverse: TATTGAATGGGCGCTAGCTT). Results were analyzed by the ΔCT method. CT values of GAPDH were subtracted from CT values of EG5. ΔCT values of treated animals were calculated as percentage of HBG treated control animals.

3.2.4. Histological evaluation

5 × 10^6 HUH7 cells were injected subcutaneously into the left flank of 6-week-old mice. When tumors reached a size of 500 mm^3, animals were divided into 6 groups (n=2) and injected intravenously into tail vein with 250 µL **1214** without HA-DBCO modification, cationic (**1214** with **0.1** equiv. of HA-DBCO modification) or anionic (**1214** with **0.8** equiv. of HA-DBCO modification) polyplexes comprising 50 µg of siCtrl spiked with 50% of Cy3-labeled siRNA at N/P 12. 5 respectively 45 min after i.v. injection the perfusion process was performed under intraperitoneal ketamine-xylazine (ketamine: 100 mg/kg, xylazine: 10 mg/kg) anesthesia. By heart puncture the animals were perfused with phosphate buffered saline (PBS), afterwards with paraformaldehyde (PFA) for 3 minutes, followed by blocking with bovine serum albumin (BSA) and staining with 50 ml DyLight 488™ labeled *Lycopersicon esculentum* lectin (20 µg/ml, diluted in PBS) [132]. In a final, step mice were perfused with BSA again. All steps were conducted for 1 minute with a flow rate of 10 ml/min and used chemicals were diluted to 1% in PBS, if not stated otherwise.

With perfusion being finished tumor and organs were harvested and cut by vibratome into 50 µm thick slices. Afterwards free-floating sections were collected from water bath, mounted with antifade mounting medium and scanned within 12 h in a Z-stack manner with 1 µm interval using a confocal microscope system. Corresponding 3D reconstruction and quantitative analysis was done using the Imaris 9.0.1 software. Perfusion and microscopic examination were performed by Mochen Cui (*PhD student at Center for Neuropathology and Prion Research, LMU*) and Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU)*.

3.3. Statistical analysis

Results are depicted as mean + S.E.M (standard error of the mean). Statistical analysis was evaluated with unpaired students t-test employing GraphPad Prism™. P-values smaller than 0.05 were regarded as significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = no significance).

III. RESULTS

To achieve effective siRNA delivery, an appropriate carrier system is crucial. For this purpose, our working group can resort to a library of about 1400 sequence-defined oligomers. In this section a siRNA transporter system, modified with HA as shielding and targeting agent, is assessed regarding biodistribution and especially tumor accumulation. Furthermore, gene silencing efficiency was investigated and immunostaining as well as 3D-reconstruction were performed to gain deeper understanding of *in vivo* distribution on a histological level.

After *in vitro* screening of different structures, the oligomer **1214** was chosen for further *in vivo* evaluation due to its superior stability (demonstrated by agarose gel electrophoresis) and transfection efficacy for siRNA (Jie Luo, unpublished data). The necessary surface modification via the azide function with HA-DBCO for efficient shielding and targeting is depicted in **Figure 4**. The amount of DBCO on HA is described as molar equivalents (equiv.) related to the cationic oligomer (mol/mol) and leads to the formation of cationic or anionic nanoparticles.

All experiments were performed with HUH7, a human hepatocarcinoma derived cell line that overexpresses the HA susceptible CD44 receptor.

Oligomers for all experiments were generated by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU).* Elisa Hörterer *(PhD student at Pharmaceutical Biotechnology, LMU)* assisted during the gene silencing and histology studies. The perfusion as well as the histological evaluation were performed by Mochen Cui (*PhD student at Center for Neuropathology and Prion Research, LMU*) and Jie Luo. All experiments were performed in NMRI nude mice.

1. *In vivo* biodistribution studies

Because a multitude of parameters influences biodistribution of siRNA polyplexes, near infrared imaging (NIR) was conducted to analyze distribution regarding tumor accessibility.

CD44 receptor positive HUH7 tumor cells were used and mice were injected

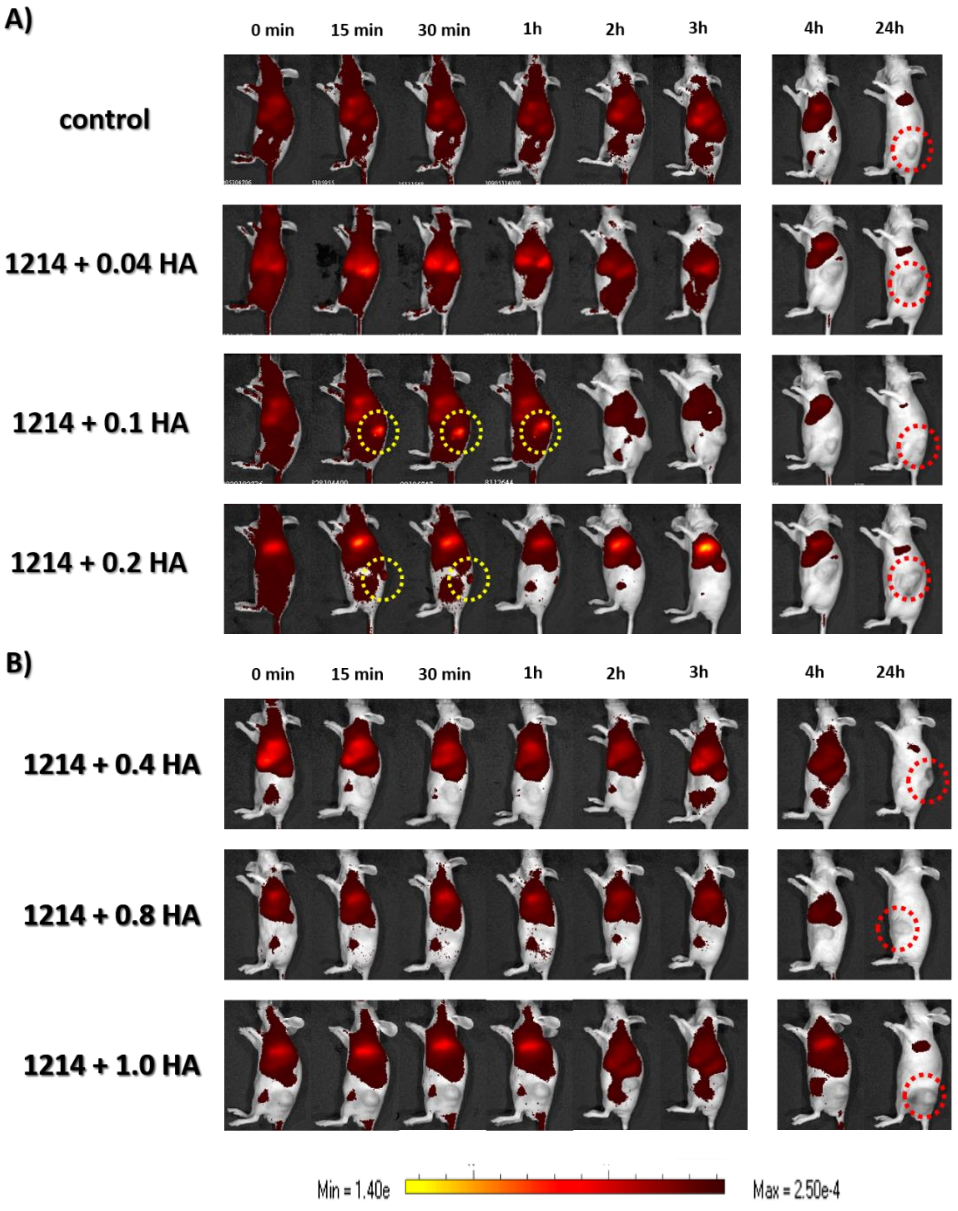
i.v. with **1214** formulations at a tumor size of 500 mm³. **1214** formulations contained 50 µg of 50% Cy7-labeled siRNA and the following equiv. of HA-DBCO: **0.04**, **0.1**, **0.2**, **0.4**, **0.8** and **1.0**. Validated by zeta potential measurement (Jie Luo, unpublished data), equiv. of HA-DBCO of **0.04**, **0.1** and **0.2** result in a cationic net charge, whereas higher amounts of HA lead to negatively charged particles. **1214** without HA-DBCO modification served as control.

After i.v. injection, fluorescent Cy7 dye was observed via *in vivo* imaging of isoflurane anesthetized mice. Eight time points were recorded up to 24 h post injectionem and additionally organs and tumor were imaged *ex vivo* after euthanasia and subsequent extraction.

Figure 6 shows the biodistribution of formulations. In comparison to the control group the cationic nanoparticles with equiv. of **0.04** up to **0.2** of HA-DBCO (illustrated in **Figure 6, A**) featured similar distribution pattern. For the equiv. of **0.04** HA slighty stronger signal is visible after 2 and 3h, but all cationic formulations exhibit comparably weak fluorescence after 4 and 24h. It is noteworthy that only the equiv. of **0.1** and **0.2** HA-DBCO modification display a signal in tumor tissue up to 1h (indicated by **yellow** circle), while the higher HA concentration of **0.2** equiv. achieves tumor accumulation only in one out of three mice and overall tumor signal is significantly weaker. For later timepoints (> 1h) the tumor signal vanishes without resurfacing (indicated by **red** circle). These findings coincide with the *ex vivo* images (illustrated in **Figure 6, C,** bottom row) that show no fluorescence signal in harvested tumor tissue (indicated by **red** circle).

On the contrary, the negatively charged formulations of **1214** modified with equiv. of **0.4** up to **1.0** of HA-DBCO (illustrated in **Figure 6, B**) demonstrate a diminished overall full-body fluorescence signal with minor differences in between the three groups, compared to animals injected with **1214** without HA modification and the cationic formulations, but nevertheless a strong signal in the lung area, equal or even slightly more intense than in the cationic formulations at the same timepoint, is visible after 4h. Consequently, for anionic particles no accumulation in tumor tissue is detectable *in vivo* or *ex vivo* (indicated by **red** circle in **Figure 6, B** and **C**).

In sum, cationic formulations evinced slightly stronger full-body fluorescence signal compared to their negatively charged counterparts. Additionally, two cationic formulations were detectable in the tumor area, but eliminated relatively fast after 1h. Regarding tumor targeting, in the direct comparison of **0.1** to **0.2** equiv. of HA-DBCO, the lower cationic concentration of **0.1** equiv. of HA proved to be superior by exhibiting stronger tumor signal in more (2 out of 3) mice up to 1h. Consistent with these findings, the *ex vivo* images taken after 3h did not show signs of intratumoral fluorescence.



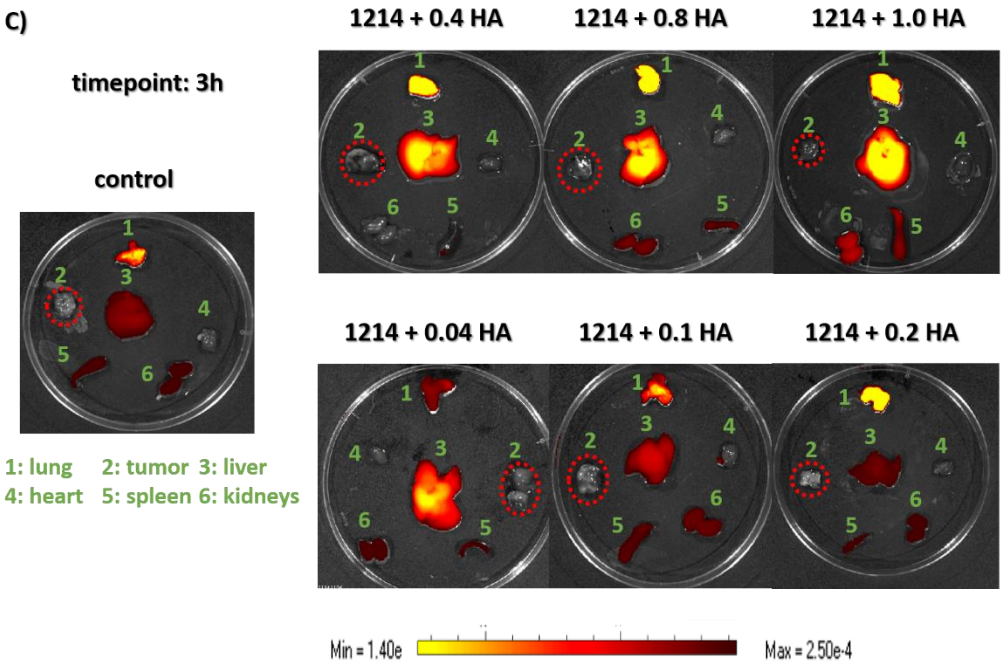


Figure 6: Distribution of polyplexes in HuH7 tumor bearing mice. NIR fluorescence bioimaging of polyplexes of **1214** containing 50% Cy7-labeled siRNA with varying equiv. of HA-DBCO modification and without HA-DBCO modification as control, was performed. Animals were imaged immediately after i.v. injection and additional images were taken after 15 and 30 min and 1, 2, 3, 4 and 24 h. In each group all 3 animals were measured up to 1 h, but only two mice were imaged after 2 and 3 h, whereas only one mouse after 4 and 24 h. After euthanasia organs were harvested and imaged, but only timepoint 3 h is displayed. For each group and timepoint, images of one representative animal are displayed. To guarantee best visibility of the tumor site on the left flank (indicated by **red** dotted circles) animals are presented in lateral position. **Yellow** dotted circles are used for images with strong signal in area of tumor site, indicating successful delivery of formulation into tumor tissue. Color scale (efficiency) had a minimum of 1.4 e^{-5} and a maximum of 2.5 e^{-4} fluorescent photons/incident excitation photon. **A)** Control and cationic formulations **B)** Anionic formulations **C)** *Ex vivo* images of organs after **3h**: 1: lung, 2: tumor (indicated by **red** dotted circles), 3: liver, 4: heart, 5: spleen, 6: kidneys.

2. Clinical blood parameters

Due to excessive accumulation in tissue, other than tumor, undesired side effects can occur. Therefore, to register the biocompatibility and exclude immune response of the siRNA polyplexes after systemic application, blood was tested for kidney and liver parameters, as they might indicate unwished adverse effects, due to their involvement in drug degradation and elimination.

The values of liver parameters AST (aspartate aminotransferase) and ALT (alanine aminotransferase) and kidney parameters BUN (blood urea nitrogen) and creatinine (illustrated in **Figure 7**) are unobtrusive, suggesting that the formulations were tolerated well by the mice. They are within normal

range, compared to the control group, and are in accordance with values in literature and the standard values provided by the supplier, Janvier [133].

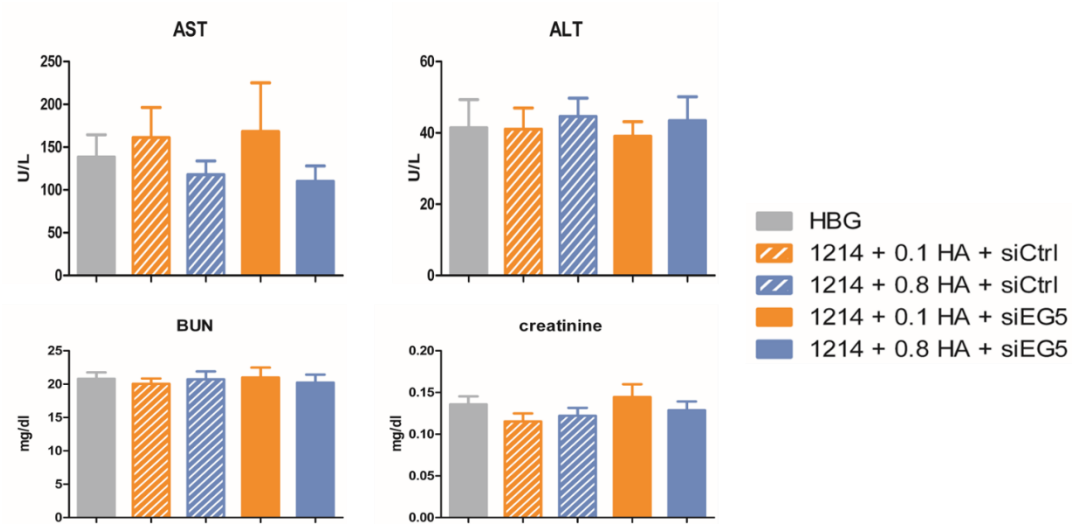


Figure 7: Clinical blood parameters. Blood was collected at the end of the gene silencing experiment from mice injected with **1214** with **0.1** equiv. of HA-DBCO modification and **1214** with **0.8** equiv. of HA-DBCO modification containing siCtrl or siEG5. HBG served as an untreated control group. The plasma extraction was followed by analysis for liver (AST, ALT) and kidney (BUN, creatinine) parameters in the *Clinic of Small Animal Medicine, Centre for Clinical Veterinary Medicine, Faculty of Veterinary Medicine, LMU Munich*. Depicted is the mean + S.E.M. of 5 mice per group.

3. *In vivo* gene silencing study

To research specific uptake into tumor cells, biodistribution imaging via Cy7 fluorescence is insufficient. For that reason, a gene silencing experiment was performed to investigate, whether delivery into the cytoplasm and subsequent target gene knockdown is possible. In this follow-up experiment the gene silencing capacity of promising anionic and cationic formulations was assessed. As cationic respectively anionic representatives **1214** modified with **0.1** respectively **0.8** equiv. of HA-DBCO were selected, since the cationic formulation exhibited tumor accumulation until 1 h after i.v. injection, while no anionic formulation could reach tumor tissue, but still **0.8** equiv. of HA-DBCO was most convincing regarding *in vitro* data (Jie Luo, unpublished data). Polyplexes were formulated with siRNA directed against the antitumoral model gene EG5. Targeting of *EG5,* which is a kinesin-related motor protein gene participating in the self-assembly of the microtubule-based mitotic spindle [134], leads to the arrest of cell mitosis. The control group was injected with HBG.

Animals were inoculated with HUH7 cells and split into 5 groups (n=5), as soon as tumors reached a size of 500 mm³. They received twofold i.v. injections into the tail vein with one day break between the administrations. 24 h after the second treatment, mice were euthanized and tumors were removed for subsequent RNA isolation and qRT-PCR analysis of mRNA levels of EG5. EG5 mRNA level of treated mice is shown in comparison to untreated HBG group (depicted in **Figure 8**).

Tumoral EG5 mRNA expression was significantly downregulated (~80%) after injection of positively charged **1214** modified with **0.1** equiv. of HA-DBCO + siEG5. The anionic formulation of **1214** modified with **0.8** equiv. of HA-DBCO + siEG5 provoked only minor EG5 gene knockdown of about 10%. For both, the anionic and the cationic formulation, the analog control formulation containing siCtrl did negligibly influence mRNA levels (depicted in **Figure 8)**.

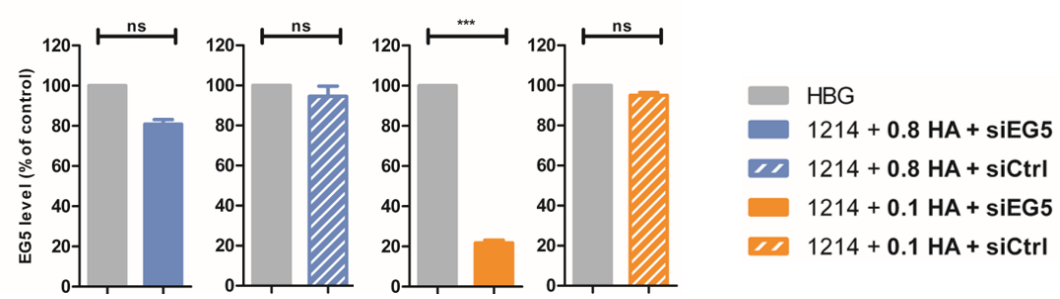


Figure 8: Gene silencing efficiency of cationic and anionic 1214 formulations in HUH7 tumor bearing mice. Groups (n=5) were injected with **1214** with **0.1** respectively **0.8** equiv. of HA-DBCO modification containing siEG5 or siCtrl. Animals were euthanized 24 h after second i.v. injection and tumors were analyzed by qRT-PCR for mRNA levels of *EG5* gene. *EG5* gene level (mean + S.E.M.) is expressed in % of untreated HBG control, defined as 100%. qRT-PCR was performed by Jie Luo (*PhD student at Pharmaceutical Biotechnology, LMU*).

Additionally, the animal weight was measured daily to monitor animal well-being, especially during treatments. No severe weight loss and therefore no negative effect of the injections could be detected.

To sum this up, only the cationic formulation of **1214** modified with **0.1** equiv. of HA-DBCO + siEG5 did significantly hamper mRNA expression, while no negative side effects could be observed via clinical biochemistry parameters and body weight.

4. Histological evaluation

Apart from proving the successful gene delivery via PCR to determine EG5 mRNA expression, analysis on a histological level can provide information about charge dependent tumoral distribution patterns. In the related experiment two fluorescent dyes were employed: DyLight 488™ that specifically stains endothelial cells of blood vessels, and Cy3 dye for exact particle identification. At 5 respectively 45 min after intravenous injection of unmodified **1214**, **1214** modified with **0.1** or **0.8** equiv. of HA-DBCO labeled with 50 µg of siCtrl spiked with 50% of fluorescent dye Cy3, into two mice in each group, perfusion and histological tissue preparation were performed according to method **3.2.5**. Analysis and processing were done by confocal laser scanning microscopy (CLSM) combined with Imaris software. The distribution of cationic and anionic HA polyplexes of **1214** is shown in **Figure 9**.

After 5 min, only the cationic formulation containing **0.1** equiv. of HA-DBCO exhibits significant endothelial attachment in tumor vessels (Cy3 labeled particles are depicted in **red**, endothelial cells of vessels in **green**), whereas the anionic formulation of **0.8** HA respectively the control group without HA modification show next to no respectively rather weak intravascular Cy3 expression. Looking at the merged image of the positively charged formulation of **1214** containing **0.1** equiv. of HA-DBCO, it becomes obvious by macroscopic inspection that the vast majority of particles seems to remain within tumor vessels.

On the contrary, the later timepoint of 45 min for **1214** containing **0.1** equiv. of HA-DBCO reveals a shift from localization mainly inside the vessels towards surrounding tissue. After 45 min the majority of particles was able to pass the vessel wall and penetrate into tumor tissue.

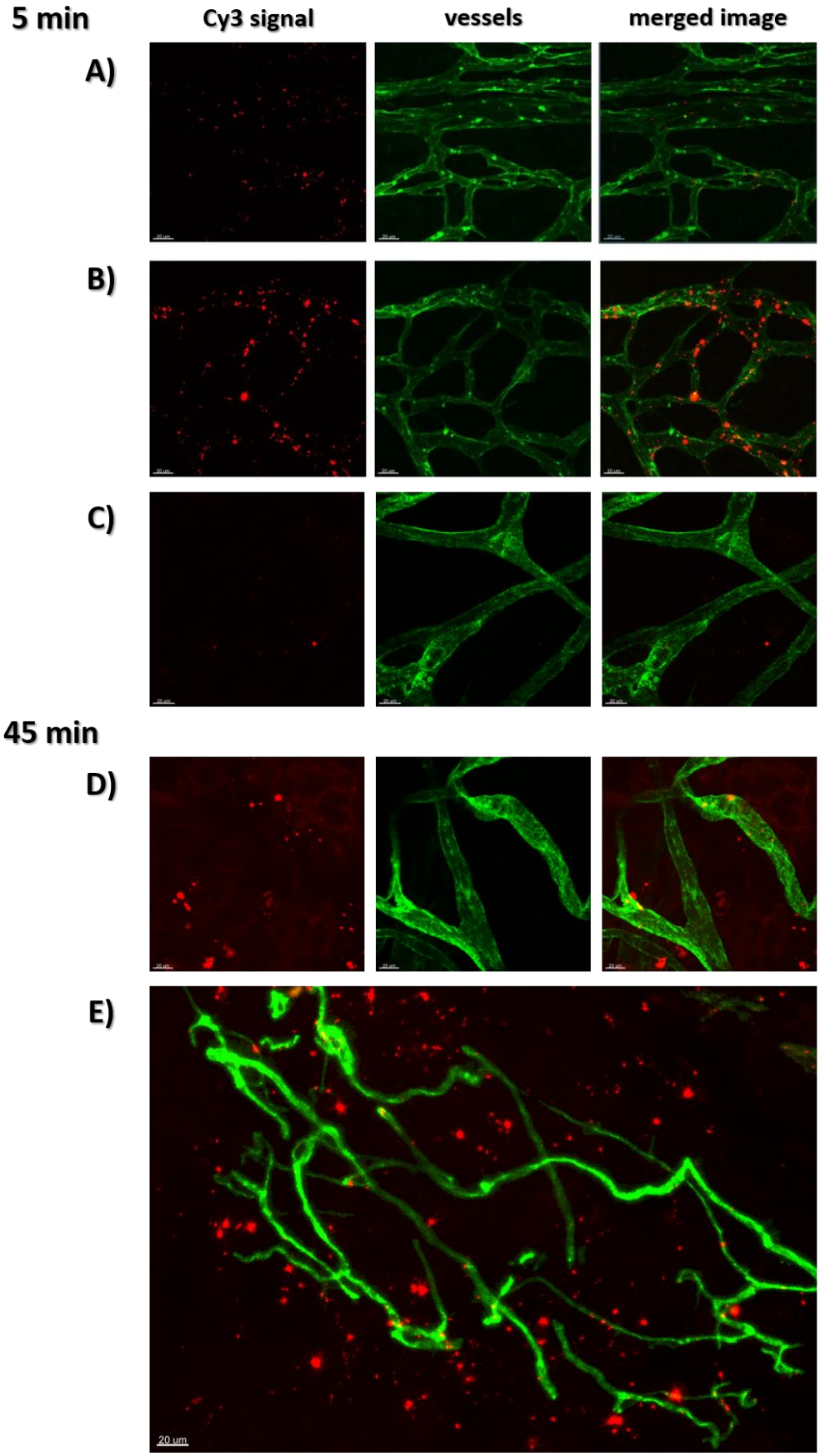


Figure 9: Distribution of cationic and anionic 1214 polyplexes in HUH7 tumors on a histological level. Six Groups (n=2) were injected with unmodified **1214** (**A**), **1214** modified with **0.1** (**B, D, E**) or **0.8** (**C**) equiv. of HA-DBCO labeled with 50 µg of siCtrl spiked

with 50% of fluorescent dye Cy3 (exhibiting **red** fluorescence signal). 5 (**A, B, C**) respectively 45 min (**D, E**) after application, perfusion, involving DyLight 488™ labeled *Lycopersicon esculentum* tomato lectin (exhibiting **green** fluorescence signal) for staining of vessels [132], was done. For each formulation the fluorescence of Cy3 and DyLight 488™ after 5 min (**A, B, C**) is depicted, as well as a merged image combing both fluorescences. For the later timepoint of 45 min (**D, E**), only **1214** modified with **0.1** equiv. of HA-DBCO is presented. This is due to the fact that it is the only formulation being present in tumor tissue in a relevant dose after 5 min (**B**). The scale bar indicates 20 µm. The figure is adapted from Luo et al., manuscript in preparation.

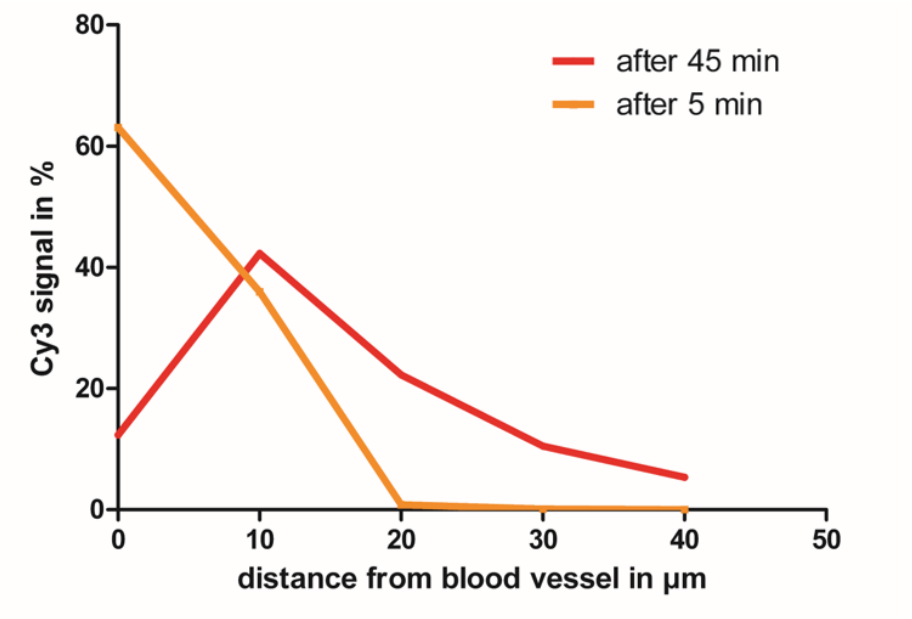


Figure 10: Quantitative data analysis of tumor tissue for cationic formulation. This illustration depicts the distance of particles of **1214**, modified with **0.1** equiv. of HA-DBCO and labeled with Cy3, from tumoral blood vessels to investigate time-dependent distribution after 5 (**orange** curve) and 45 (**red** curve) min. The y-axis presents the percentage of Cy3 signal compared to a total signal of 100%. The figure is adapted from Luo et al., manuscript in preparation.

Analysis of quantitative distribution data (illustrated in **Figure 10**) reveals that with elapsing time the Cy3 signal in tumor tissue increases, while it declines inside respectively in close proximity to blood vessels. After 5 min more than 60% of the nanoparticles are located inside the tumor vessels, whereas after 45 min less than 20% can be found intravascular and the largest part (> 80%) are at least 10 µm away from vessels.

In conclusion, relevant accumulation in tumor vessels and tissue was only obtained by the cationic formulation of **1214** with HA, while the control group and the anionic counterpart could not achieve tumor delivery, which is in

accordance with the findings of the biodistribution studies and the gene silencing experiment. In addition, the tumoral distribution for **0.1** equiv. of HA develops over time, resulting in an increased nanoparticle concentration in tissue compared to blood vessels after 45 min.

IV. DISCUSSION

With the first siRNA drugs being approved for hereditary ATTR amyloidosis and acute intermittent porphyria (AIP) and many more currently undergoing clinical trials [135, 136], the potential of siRNA-based therapies is evidenced. Nevertheless delivery of siRNA into target tissue presents an obstacle, due to its poor pharmacokinetics [137-139]. A solution to this issue is effective carrier systems to increase stability and by that enhance blood circulation time, as well as achieve specific targeting to desired tissues. Our working group established a library of about 1400 sequence-defined oligomers that can serve as transporters for nucleic acids like siRNA. Their advantages lie in reduced particle size and masking the positively charged backbone, therefore limiting cell toxicity [17]. Several recent studies underline the potential of these carriers concerning nucleic acid transportation [106, 108, 111, 118]. Still, stability and renal excretion after systemic application represent great hurdles for these carriers that could be solved by continuous revision and improvement with beneficial features like surface functionalization (e.g. by employing click-chemistry).

The oligomer **1214** was selected for siRNA delivery to a subcutaneous tumor model after intravenous injection. Furthermore, modification with hyaluronic acid (HA) aimed at tumor tissue selective accumulation by passive and active targeting with constant biocompatibility.

1. Tumor targeting via biodistribution studies

PEG is used for the last three decades to prolong circulation time in blood by protecting the nanoparticles from quick elimination by the kidney and adverse immune reactions, both resulting in decreased delivery and accumulation in target tissue [63, 67]. An alternative shielding agent, the natural polysaccharide HA [78-80] that does not exhibit pronounced immunogenic reactions, could additionally serve as an active targeting ligand by binding to the receptor CD44 [127-129]. The required overexpression of CD44 is reported for many tumoral cell lines (e.g. HUH7) and could assist in tumor selective delivery. Via an azide function as a functional domain the oligomer can subsequently be modified with

cyclooctyne derivates (e.g. DBCO) [140] and by that smoothly functionalize the polyplex via click-chemistry. Other functional groups like thiols show promising gene silencing efficacy *in vitro*, but *in vivo* present disadvantages mainly regarding stability [117, 141, 142], while cyclooctynes can react with azide groups enabling efficient coupling without by-products and copper related cytotoxicity [51, 52, 143].

Hence, oligomeric formulations of **1214** modified by click-chemistry with differing amounts of HA were evaluated in a biodistribution experiment for this thesis.

HA allowed efficient shielding of the oligomer and the linked siRNA, indicated by significant signal after 24h. Although *in vitro* agarose gel electrophoresis (Jie Luo, unpublished data) suggested equal stability for all anionic and cationic particles, with amounts bigger than **0.2** equiv. of HA-DBCO modification, the result *in vivo* is weaker full-body biodistribution. This might be due to decreased *in vivo* stability of the anionic formulations, because of a critical threshold of HA that was exceeded. **0.1** and **0.2** equiv. of HA-DBCO seem to represent the ideal amount of HA, which can effectively shield the cationic backbone from negatively charged blood components and thereby facilitate significant retention in tumor tissue. However, this objects previous efforts to create anionic nanoparticles to circumvent rapid plasma clearance involving undesired interactions with blood components. But apart from negative charge other factors like the ability to penetrate capillaries of other tissues, the hepatic metabolization of the nanoparticles and the renal clearance of degradation products, play a role in the elimination from plasma [144]. Our biodistribution experiments demonstrated that overall cationic charge is tolerable, if the shielding agent can partly hide the cationic charge through its anionic character. This partly shielding enables accumulation in tumor tissue with **0.1** equiv. of HA displaying strongest direction to site of action. Nevertheless, comparably fast tumoral clearance resulting in no tumor signal after 2 h, indicates that necessary efforts have to be made regarding further modification of the oligomer to prolong tumoral retention. Furthermore, biodistribution only gives information about tumor accumulation, but ignores exact siRNA location and functionality.

2. Gene silencing efficiency *in vivo*

The biodistribution experiments present the effective usage of HA for surface shielding after systemic administration. Due to passive targeting e.g. via the EPR effect by adequate nanoparticle size [145], enhanced blood circulation and permeable vessels next to diminished clearance by the lymph system, tumoral delivery is possible [146]. After targeting the site of action, the active substance has to be delivered into the cytosol. The actual uptake into tumor cells can be directed via active targeting of the receptors, e.g. CD44. In the presented experiments, the HA modified nanoparticles can selectively bind to solid HUH7 tumors overexpressing CD44 [147] and internalize via receptor-mediated endocytosis.

The formulation of **1214** with varying equiv. of HA-DBCO was examined regarding its potential to silence the target gene EG5. For that purpose, siRNA directed against EG5 mRNA was introduced. Eglin 5 or kinesin spindle protein (KSP), the so-called EG5, plays a vital role in mitosis during the self-assembly of the microtubule-based spindle [148]. Although it is a valuable target in cancer therapy, due to its knockdown leading to inhibition of cell division and finally cell death, in our study it only served as a marker gene to register successful internalization and release into cytoplasm of siRNA integrated into nanoparticles with subsequent reduced expression of the target gene EG5.

Directing **1214** with **0.1** equiv. of HA-DBCO towards EG5, resulted in a gene silencing effect of about ~80%, surpassing the efficiency of ~60% respectively 65% of previous studies [108, 111]. Conversely, the anionic formulation with **0.8** equiv. displayed EG5 gene expression of about 90%, being tantamount with a gene knockdown of only ~10%. The analog siCtrl formulations showed only minor reduction of EG5 expression making unspecific gene silencing by the polyplexes themselves highly unlikely. The data suggests that the cationic HA formulation was able to enter tumor cells more effectively and therefore deliver the complexed siRNA into the cytosol resulting in significant EG5 gene knockdown. In combination with the findings from the biodistribution studies, we can state that the anionic formulation, including **0.8** equiv. of HA-DBCO, is explicitly inferior concerning tumor delivery and conditional siRNA uptake and cytosolic

release into HUH7 cells. The novel assumption that uptake into tumor tissue is an active process mediated by endothelial cells [33] might be an explanation. Attachment to the negatively charged glycocalyx of endothelial cells [149] is a prerequisite that can be met by cationic nanoparticles more easily [85]. Another hypothesis is the binding of cationic nanoformulations to anionic lymphocytes and subsequent transportation into tumor tissue [150-152]. Though anionic net charge is considered advantageous regarding parameters like circulation time and shows comparable transfection efficacy *in vitro*, the cationic formulation prevails in our experiments. This can result from increased tumor accumulation because of its cationic charge leading to increased trans-endothelial uptake into solid tumor tissue. Additionally, HA induces receptor-mediated uptake into CD44 positive HUH7 cells [147], which can only function for the **0.1** equiv. of HA, due to the fact it can be determined in relevant levels in tumor tissue, in contrast to the anionic formulation.

The inspection on a histological level specifies the biodistribution studies by demonstrating the connection between cationic charge and improved tumoral uptake, whereas equiv. of **0.8** HA feature only negligible tumoral signal. Interestingly, with time elapsing the intravascular nanoparticle concentration reduces, while the portion in surrounding tumor tissue rises. While this finding cannot be derived from the previous experiments, it makes sense that a time dependent distribution (e.g. after 45 m) is ascertainable. Furthermore, the histological examination underlines the hypothesis that tumor accumulation of cationic particles is mediated after attachment to endothelial cells.

Also, biochemical determination of important renal and liver parameters and regular weight determination indicate biocompatibility and show no signs for acute toxicity or dysfunction of liver or kidney after formulation application.

Further experiments should be performed to investigate the effect in various cell lines and for enhanced transporter systems for efficient delivery and prolonged blood circulation. Moreover, the relevance of the deployed carrier in therapeutic studies should be assessed.

V. SUMMARY

Cancer poses a leading cause for human death worldwide, with numbers rising, due to population ageing. Apart from medical causes, economic reasons urge for novel treatment approaches. Current treatment options lack efficiency, especially for metastasizing non-operable tumors, while prophylaxis presents a challenge, because of multifactorial pathogenesis. Extensive research in this field, put forth siRNA as an innovative therapeutic option with hepatocyte-targeted Patisiran (Onpattro™) and Givosiran (Givlaari™) being approved for medical application. Still, intratumoral siRNA delivery faces multiple obstacles.

Hence, this thesis aimed at researching hyaluronic acid (HA) shielded and targeted sequence-defined oligomeric carriers for specific *in vivo* delivery of siRNA into a subcutaneous human tumor xenograft mouse model after intravenous administration.

In the first part a biodistribution study revealed that only cationic formulations managed to reach subcutaneous hepatocellular HUH7 carcinoma tissue until 1 h after injection, while for the control group and higher HA levels, resulting in negatively charged particles, no tumor targeting was achieved. A follow up gene silencing study combined cationic and anionic formulations with siRNA directed towards the mitosis associated EG5 gene. Systemic application lead to a significant target gene knockdown of ~80% for cationic particles, comprising **0.1** equiv. of HA, whereas anionic nanoparticles did not significantly reduce gene expression in subcutaneous HUH7 tumors. For cationic polyplexes subsequent histological evaluation evinced efficient binding to endothelial cells in tumor vessels followed by transport into surrounding tissue, while only a negligible quantity of anionic nanoparticles reached tumoral vessels in the first place.

Polyplexes modified via click-chemistry with surface shielding and targeting ligand HA presented good tolerability after intravenous injection into mice, indicated by biochemistry parameters and animal weight being within normal range and without significant deviation.

In sum, a HA-shielded and targeted nanocarrier was demonstrated as an

efficient system for siRNA delivery into tumor tissue and subsequent gene silencing. These promising results are encouraging regarding further studies to develop and enhance siRNA cancer therapeutics.

VI. ZUSAMMENFASSUNG

In vivo Evaluierung polymerischer siRNA Nanoformulierungen für die tumorale Genexpressionshemmung

Weltweit stellt Krebs bei Menschen eine häufige Todesursache mit steigenden Fallzahlen aufgrund des zunehmenden Durchschnittsalters dar. Neben medizinischen gibt es wirtschaftliche Gründe für neuartige Behandlungsansätze. Aktuellen Behandlungsmöglichkeiten fehlt es an Wirksamkeit, vor allem bei metastasierenden nicht operablen Tumoren, während Vorbeugemaßnahmen, aufgrund der multifaktoriellen Krankheitsentstehung, eine Herausforderung darstellen. Umfangreiche Forschung auf diesem Gebiet hat siRNA als bahnbrechende Therapiemöglichkeit hervorgebracht. Patisiran (Onpattro^{TM}) und Givosiran (Givlaari^{TM}), beide für Leber-spezifische RNA Interferenz entwickelt, wurden bereits für den medizinischen Einsatz zugelassen. Dennoch muss für den Transport von siRNA in Tumorgewebe eine Vielzahl von Hürden überwunden werden.

Daher war es das Ziel dieser Arbeit mit Hyaluronsäure (HA) abgeschirmte und ausgerichtete sequenzdefinierte Träger-Oligomere hinsichtlich ihrer spezifischen Einschleusung von siRNA in Tumoren nach systemischer Verabreichung im Xenograft Mausmodell zu untersuchen.

Im ersten Teil der Arbeit ergab ein Biodistributionsversuch, dass kationische Formulierungen in der Lage waren subkutane hepatozelluläre HUH7 Tumore für bis eine Stunde zu erreichen, während die Kontrollgruppe bzw. höhere HA-Konzentrationen, die negative geladene Teilchen zur Folge haben, sich nicht im Tumor anreichern konnten. Im Folgeversuch, der die Hemmung der Genexpression untersucht, wurden kationische und anionische Formulierungen mit siRNA kombiniert, die sich gegen das an der Mitose beteiligte EG5 Gen richtet. Die systemische Gabe der kationischen Teilchen, die **0.1** Äquivalente von HA enthalten, führte zu einer signifikanten Herunterregulierung des Zielgens von ungefähr 80%, wohingegen die anionischen Nanopartikel die Genexpression in subkutanen HUH7-Tumoren nicht signifikant verminderten. Die nachfolgende histologische

Untersuchung zeigte, dass kationische Polyplexe an die Endothelzellen der Tumorgefäße binden und danach in das umgebende Tumorgewebe transportiert werden, während anionische Formulierungen überhaupt nur in vernachlässigbarer Menge den Tumorgefäßen ankommen.

Die mittels “Klick-Chemie” mit dem abschirmenden Targeting-Ligand HA modifizierten Polyplexe zeigen nach intravenöser Injektion in Mäusen gute Verträglichkeit. Dies deuten die biochemischen Parameter sowie das Tiergewicht an, die innerhalb des Normalbereichs liegen und keinerlei signifikante Abweichungen zeigen.

Summa summarum wurde ein mittels Hyaluronsäure abgeschirmtes und zielgerichtetes Trägersystem für den effektiven Transport von siRNA in Tumorgewebe mit sich anschließender Genexpressionshemmung etabliert. Die vielversprechenden Ergebnisse machen Mut hinsichtlich weiterer Studien für die Entwicklung und Verbesserung siRNA-basierter Krebstherapeutika.

VII. REFERENCES

1. Wild, C.P., Weiderpass, E., Stewart, B. W., *World Cancer Report.* 2020.
2. Zentrum für Krebsregisterdaten und Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V., *Krebs in Deutschland für 2015/2016.* 2019.
3. WHO. *Cancer*. 2018 [cited 2020 05 february]; Available from: https://www.who.int/news-room/fact-sheets/detail/cancer.
4. Yeldag, G., Rice, A., and Del Río Hernández, A., *Chemoresistance and the Self-Maintaining Tumor Microenvironment.* Cancers, 2018. **10**(12): p. 471.
5. Li, S. and Huang, L., *Nonviral gene therapy: promises and challenges.* Gene Therapy, 2000. **7**(1): p. 31-34.
6. The Cancer Genome Atlas Research Network, *Integrated genomic analyses of ovarian carcinoma.* Nature, 2011. **474**(7353): p. 609-615.
7. Sameek, R. and Chinnaiyan, A.M., *Translating Genomics for Precision Cancer Medicine.* Annual Review of Genomics and Human Genetics, 2014. **15**(1): p. 395-415.
8. Liu, Y., Xu, C.-F., Iqbal, S., Yang, X.-Z., and Wang, J., *Responsive Nanocarriers as an emerging platform for cascaded delivery of nucleic acids to cancer.* Advanced Drug Delivery Reviews, 2017.
9. Zhang, G., Budker, V., and Wolff, J.A., *High Levels of Foreign Gene Expression in Hepatocytes after Tail Vein Injections of Naked Plasmid DNA.* Human Gene Therapy, 2004. **10**.
10. Bartlett, D.W. and Davis, M.E., *Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging.* Nucleic Acids Research, 2006. **34**(1): p. 322–333.
11. Linnane, E., Davey, P., Zhang, P., Puri, S., Edbrooke, M., Chiarparin, E., Revenko, A.S., Macleod, A.R., Norman, J.C., and Ross, S.J., *Differential uptake, kinetics and mechanisms ofintracellular trafficking of next-generation antisenseoligonucleotides across human cancer cell lines.* Nucleic Acids Research, 2019. **47**(9): p. 4375–4392.
12. Wagner, E., *Biomaterials in RNAi therapeutics: quo vadis?* Biomaterials Science, 2013. **1**(8): p. 804.
13. Chou, S.-T. and Mixson, A.J., *siRNA nanoparticles: the future of RNAi therapeutics for oncology?* Nanomedicine, 2014. **9**(15): p. 2251-2254.
14. Davis, M.E., Zuckerman, J.E., Choi, C.H.J., Seligson, D., Tolcher, A., Alabi, C.A., Yen, Y., Heidel, J.D., and Ribas, A., *Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles.* Nature, 2010. **464**(7291): p. 1067-1070.
15. Scott, D.W. and Lozier, J.N., *Gene therapy for haemophilia: prospects and challenges to prevent or reverse inhibitor formation.* British Journal of Haematology, 2012. **156**(3): p. 295-302.
16. Ameres, S.L., Martinez, J., and Schroeder, R., *Molecular Basis for Target RNA Recognition and Cleavage by Human RISC.* 2007. **130**(1): p. 101-112.
17. Lächelt, U. and Wagner, E., *Nucleic Acid Therapeutics Using*

Polyplexes: A Journey of 50 Years (and Beyond). Chemical Reviews, 2015. **115**(19): p. 11043-11078.
18. FDA. *FDA approves first-of-its kind targeted RNA-based therapy to treat a rare disease* 2018 [cited 2020 06 february]; Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-first-its-kind-targeted-rna-based-therapy-treat-rare-disease.
19. Ratner, M., *Patients with porphyria bask in sunlight of FDA approval.* Nature Biotechnology, 2019. **37**(12): p. 1390-91.
20. FDA. *FDA approves givosiran for acute hepatic porphyria*. 2019 [cited 2020 09 february]; Available from: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-givosiran-acute-hepatic-porphyria.
21. Dominska, M. and Dykxhoorn, D.M., *Breaking down the barriers: siRNA delivery and endosome escape.* Journal of Cell Science, 2010. **123**(8): p. 1183-1189.
22. Wilhelm, S., Tavares, A.J., Dai, Q., Ohta, S., Audet, J., Dvorak, H.F., and Chan, W.C.W., *Analysis of nanoparticle delivery to tumours.* Nature Reviews Materials, 2016. **1**(5): p. 16014.
23. Haussecker, D., *Current issues of RNAi therapeutics delivery and development.* Journal of Controlled Release, 2014. **195**: p. 49-54.
24. Naeye, B., Deschouta, H., Caveliers, V., Descamps, B., Braeckmans, K., Vanhove, C., Demeester, J., Lahoutte, T., De Smedt, S.C., and Raemdonck, K., *In vivo disassembly of IV administered siRNA matrix nanoparticles at the renal filtration barrier.* Biomaterials, 2013. **34**(9): p. 2350 - 2358.
25. Takakura, Y., Nishikawa, M., Yamashita, F., and Hashida, M., *Development of gene drug delivery systems based on pharmacokinetic studies.* European Journal of Pharmaceutical Sciences, 2001. **13**(1): p. 71-76.
26. Lorenzer, C., Dirin, M., Winkler, A.-M., Baumann, V., and Winkler, J., *Going beyond the liver: Progress and challenges of targeted delivery of siRNA therapeutics.* 2015. **203**: p. 1-15.
27. Moghimi, S., Hunter, A., and Murray, J., *Long-circulating and target-specific nanoparticles: theory to practice.* Pharmacol Rev. , 2001. **53**(2): p. 283-318.
28. Plank, C., Mechtler, K., SzokaJ., F.C., and Wagner, E., *Activation of the Complement System by Synthetic DNA Complexes: A Potential Barrier for Intravenous Gene Delivery.* Human Gene Therapy, 1996. **7**.
29. Zuckerman, J.E., Choi, C.H.J., Han, H., and Davis, M.E., *Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane.* 2012. **109**(8): p. 3137-3142.
30. Smrekar, B., Wightman, L., Wolschek, M.F., Lichtenberger, C., Ruzicka, R., Ogris, M., Rödl, W., Kursa, M., Wagner, E., and Kircheis, R., *Tissue-dependent factors affect gene delivery to tumors in vivo.* 2003. **10**(13): p. 1079-1088.
31. Jain, R.K., *Transport of molecules, particles, and cells in solid tumors.* Annual review of biomedical engineering, 1999. **1**: p. 241-263.
32. Hashizume, H., Baluk, P., Morikawa, S., McLean, J.W., Thurston, G., Roberge, S., Jain, R.K., and McDonald, D.M., *Openings between*

defective endothelial cells explain tumor vessel leakiness. The American journal of pathology, 2000. **156**(4): p. 1363-1380.
33. Sindhwani, S., Syed, A.M., Ngai, J., Kingston, B.R., Maiorino, L., Rothschild, J., Macmillan, P., Zhang, Y., Rajesh, N.U., Hoang, T., Wu, J.L.Y., Wilhelm, S., Zilman, A., Gadde, S., Sulaiman, A., Ouyang, B., Lin, Z., Wang, L., Egeblad, M., and Chan, W.C.W., *The entry of nanoparticles into solid tumours.* Nature Materials, 2020.
34. Chen, Y.H., Keiser, M.S., and Davidson, B.L., *Viral Vectors for Gene Transfer.* Current Protocols in Mouse Biology, 2018. **8**(4): p. e58.
35. Daniel, R. and Smith, J.A., *Integration Site Selection by Retroviral Vectors: Molecular Mechanism and Clinical Consequences.* Human Gene Therapy, 2008. **19**(6): p. 557-568.
36. Blessing, D., Déglon, N., and Schneider, B.L., *Scalable Production and Purification of Adeno-Associated Viral Vectors (AAV)*. 2018, Springer New York. p. 259-274.
37. Yin, H., Kanasty, R.L., Eltoukhy, A.A., Vegas, A.J., Dorkin, J.R., and Anderson, D.G., *Non-viral vectors for gene-based therapy.* Nature Reviews Genetics, 2014. **15**(8): p. 541-555.
38. Felgner, P.L., Barenholz , Y., Behr, J.P., Cheng, S.H., Cullis, P., Huang, L., Jessee, J.A., Seymour, L., Szoka, F., Thierry, A.R., Wagner, E., and Wu, G., *Nomenclature for Synthetic Gene Delivery Systems.* Human Gene Therapy, 1997. **8**: p. 511–512.
39. Ogris, M., Steinlein, P., Carotta, S., Brunner, S., and Wagner, E., *DNA/polyethylenimine transfection particles: Influence of ligands, polymer size, and PEGylation on internalization and gene expression.* AAPS PharmSci, 2001. **3**(3): p. 43-53.
40. Clement, J., Kiefer, K., Kimpfler, A., Garidel, P., and Peschka-Süss, R., *Large-scale production of lipoplexes with long shelf-life.* European Journal of Pharmaceutics and Biopharmaceutics, 2005. **59**(1): p. 35-43.
41. Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., and Kissel, T., *In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis.* Biomaterials, 2003. **24**(7): p. 1121-1131.
42. Lächelt, U., *Proton-sponge activity and receptor-targeting of sequence-defined nucleic acid carriers.* LMU, 2014.
43. Sun, Y., Li, Y., Nan, S., Zhang, L., Huang, H., and Wang, J., *Synthesis and characterization of pH-sensitive poly(itaconic acid)–poly(ethylene glycol)–folate–poly(l-histidine) micelles for enhancing tumor therapy and tunable drug release.* Journal of Colloid and Interface Science, 2015. **458**: p. 119-129.
44. Hartmann, L., Krause, E., Antonietti, M., and Börner, H.G., *Solid-Phase Supported Polymer Synthesis of Sequence-Defined, Multifunctional Poly(amidoamines).* Biomacromolecules, 2006. **7**(4): p. 1239-1244.
45. Schaffert, D., Badgujar, N., and Wagner, E., *Novel Fmoc-Polyamino Acids for Solid-Phase Synthesis of Defined Polyamidoamines.* Organic Letters, 2011. **13**(7): p. 1586-1589.
46. Dohmen, C., Edinger, D., Fröhlich, T., Schreiner, L., Lächelt, U., Troiber, C., Rädler, J., Hadwiger, P., Vornlocher, H.-P., and Wagner, E., *Nanosized Multifunctional Polyplexes for Receptor-Mediated SiRNA Delivery.* 2012. **6**(6): p. 5198-5208.

47. Salcher, E.E., Kos, P., Fröhlich, T., Badgujar, N., Scheible, M., and Wagner, E., *Sequence-defined four-arm oligo(ethanamino)amides for pDNA and siRNA delivery: Impact of building blocks on efficacy.* Journal of Controlled Release, 2012. **164**(3): p. 380-386.
48. Klein, P.M., Müller, K., Gutmann, C., Kos, P., Krhac Levacic, A., Edinger, D., Höhn, M., Leroux, J.-C., Gauthier, M.A., and Wagner, E., *Twin disulfides as opportunity for improving stability and transfection efficiency of oligoaminoethane polyplexes.* Journal of Controlled Release, 2015. **205**: p. 109-119.
49. Troiber, C., Edinger, D., Kos, P., Schreiner, L., Kläger, R., Herrmann, A., and Wagner, E., *Stabilizing effect of tyrosine trimers on pDNA and siRNA polyplexes.* Biomaterials, 2013. **34**(5): p. 1624-1633.
50. Chang, K.-L., Higuchi, Y., Kawakami, S., Yamashita, F., and Hashida, M., *Efficient Gene Transfection by Histidine-Modified Chitosan through Enhancement of Endosomal Escape.* 2010. **21**(6): p. 1087-1095.
51. Agard, N.J., Prescher, J.A., and Bertozzi, C.R., *A Strain-Promoted [3 + 2] Azide−Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems.* Journal of the American Chemical Society, 2004. **126**(46): p. 15046-15047.
52. Klein, P.M. and Wagner, E., *Click-Shielded and Targeted Lipopolyplexes.* Oligonucleotide-Based Therapies, 2019: p. 141-164.
53. Scholz, C. and Wagner, E., *Therapeutic plasmid DNA versus siRNA delivery: Common and different tasks for synthetic carriers.* Journal of Controlled Release, 2012. **161**(2): p. 554-565.
54. Fröhlich, T., Edinger, D., Kläger, R., Troiber, C., Salcher, E., Badgujar, N., Martin, I., Schaffert, D., Cengizeroglu, A., Hadwiger, P., Vornlocher, H.-P., and Wagner, E., *Structure–activity relationships of siRNA carriers based on sequence-defined oligo (ethane amino) amides.* Journal of Controlled Release, 2012. **160**(3): p. 532-541.
55. Schaffert, D., Troiber, C., Salcher, E.E., Fröhlich, T., Martin, I., Badgujar, N., Dohmen, C., Edinger, D., Kläger, R., Maiwald, G., Farkasova, K., Seeber, S., Jahn-Hofmann, K., Hadwiger, P., and Wagner, E., *Solid-Phase Synthesis of Sequence-Defined T-, i-, and U-Shape Polymers for pDNA and siRNA Delivery.* Angewandte Chemie International Edition, 2011. **50**(38): p. 8986-8989.
56. Mendes, L.P., Gaeti, M.P.N., de Ávila, P.H.M., Vieira, M.d.S., Rodrigues, B.d.S., Marcelino, R.I.d.Á., dos Santos, L.C.R., Valadares, M.C., and Lima, E.M., *Multicompartimental Nanoparticles for Co-Encapsulation and Multimodal Drug Delivery to Tumor Cells and Neovasculature.* Pharmaceutical Research 2013. **31**: p. 1106–1119.
57. Wagner, E., *Polymers for siRNA Delivery: Inspired by Viruses to be Targeted, Dynamic, and Precise.* Accounts of Chemical Research, 2012. **45**(7): p. 1005-1013.
58. Abuchowski, A., McCoy, J.R., Palczuk, N.C., Es, T.v., and Davis, F.F., *Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase* J Biol Chem, 1977. **252**(11): p. 3582-6
59. Maeda, H., *The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular*

drug targeting. Advances in Enzyme Regulation, 2001. **41**(1): p. 189-207.
60. Maeda, H., Wu, J., Sawa, T., Matsumura, Y., and Hori, K., *Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review.* Journal of Controlled Release, 2000. **65**(1-2): p. 271-284.
61. Maeda, H., *Polymer therapeutics and the EPR effect.* Journal of Drug Targeting, 2017. **25**(9-10): p. 781-785.
62. Clemons, T.D., Singh, R., Sorolla, A., Chaudhari, N., Hubbard, A., and Iyer, K.S., *Distinction Between Active and Passive Targeting of Nanoparticles Dictate Their Overall Therapeutic Efficacy.* Langmuir, 2018. **34**(50): p. 15343-15349.
63. Senior, J., Delgado, C., Fisher, D., Tilcock, C., and Gregoriadis, G., *Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: Studies with poly(ethylene glycol)-coated vesicles.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1991. **1062**(1): p. 77-82.
64. Meyer, M. and Wagner, E., *pH-responsive shielding of non-viral gene vectors.* Expert Opinion on Drug Delivery, 2006. **3**(5): p. 563-571.
65. He, D. and Wagner, E., *Defined Polymeric Materials for Gene Delivery.* Macromolecular Bioscience, 2015. **15**(5): p. 600-612.
66. Cho, K., Wang, X., Nie, S., Chen, Z., and Shin, D.M., *Therapeutic Nanoparticles for Drug Delivery in Cancer.* Clinical Cancer Research, 2008. **14**(5): p. 1310-1316.
67. Klibanov, A.L., Maruyama, K., Torchilin, V.P., and Huang, L., *Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes.* FEBS Letters, 1990. **268**(1): p. 235-237.
68. Yang, Q. and Lai, S.K., *Anti-PEG immunity: emergence, characteristics, and unaddressed questions.* Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2015. **7**(5): p. 655-677.
69. Le, T.C., Penna, M., Winkler, D.A., and Yarovsky, I., *Quantitative design rules for protein-resistant surface coatings using machine learning.* Scientific Reports, 2019. **9**(1).
70. Mendler, C.T., Friedrich, L., Laitinen, I., Schlapschy, M., Schwaiger, M., Wester, H.-J., and Skerra, A., *High contrast tumor imaging with radio-labeled antibody Fab fragments tailored for optimized pharmacokinetics via PASylation.* mAbs, 2015. **7**(1): p. 96-109.
71. Schlapschy, M., Binder, U., Borger, C., Theobald, I., Wachinger, K., Kisling, S., Haller, D., and Skerra, A., *PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins.* Protein Engineering Design and Selection, 2013. **26**(8): p. 489-501.
72. Zhang, Y., Lin, L., Liu, L., Liu, F., Maruyama, A., Tian, H., and Chen, X., *Ionic-crosslinked polysaccharide/PEI/DNA nanoparticles for stabilized gene delivery.* Carbohydrate Polymers, 2018. **201**: p. 246-256.
73. Noga, M., Edinger, D., Rödl, W., Wagner, E., Winter, G., and Besheer, A., *Controlled shielding and deshielding of gene delivery polyplexes using hydroxyethyl starch (HES) and alpha-amylase.* Journal of Controlled Release, 2012. **159**(1): p. 92-103.
74. Schneider, M., Fetsch, C., Amin, I., Jordan, R., and Luxenhofer, R.,

Polypeptoid Brushes by Surface-Initiated Polymerization of N-Substituted Glycine N -Carboxyanhydrides. 2013. **29**(23): p. 6983-6988.

75. Schneider, M., Tang, Z., Richter, M., Marschelke, C., Förster, P., Wegener, E., Amin, I., Zimmermann, H., Scharnweber, D., Braun, H.-G., Luxenhofer, R., and Jordan, R., *Patterned Polypeptoid Brushes.* 2016. **16**(1): p. 75-81.
76. Yoo, J., Birke, A., Kim, J., Jang, Y., Song, S.Y., Ryu, S., Kim, B.-S., Kim, B.-G., Barz, M., and Char, K., *Cooperative Catechol-Functionalized Polypept(o)ide Brushes and Ag Nanoparticles for Combination of Protein Resistance and Antimicrobial Activity on Metal Oxide Surfaces.* Biomacromolecules, 2018. **19**(5): p. 1602-1613.
77. Klein, P., Klinker, K., Zhang, W., Kern, S., Kessel, E., Wagner, E., and Barz, M., *Efficient Shielding of Polyplexes Using Heterotelechelic Polysarcosines.* Polymers, 2018. **10**(6): p. 689.
78. Kircheis, R., Wightman, L., Schreiber, A., Robitza, B., Rössler, V., Kursa, M., and Wagner, E., *Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application.* Gene Therapy, 2001. **8**(1): p. 28-40.
79. Kircheis, R., Wightman, L., and Wagner, E., *Design and gene delivery activity of modified polyethylenimines.* Advanced Drug Delivery Reviews, 2001. **53**(3): p. 341-358.
80. Novo, L., Mastrobattista, E., Van Nostrum, C.F., Lammers, T., and Hennink, W.E., *Decationized polyplexes for gene delivery.* Expert Opinion on Drug Delivery, 2015. **12**(4): p. 507-512.
81. Huayu, T., Lin, L., Chen, J., Chen, X., Park, T.G., and Maruyama, A., *RGD targeting hyaluronic acid coating system for PEI-PBLG polycation gene carriers.* Journal of Controlled Release, 2011. **155**(1): p. 47-53.
82. Yang, Y., Jia, Y., Xiao, Y., Hao, Y., Zhang, L., Chen, X., He, J., Zhao, Y., and Qian, Z., *Tumor-Targeting Anti-MicroRNA-155 Delivery Based on Biodegradable Poly(ester amine) and Hyaluronic Acid Shielding for Lung Cancer Therapy.* ChemPhysChem, 2018. **19**(16): p. 2058-2069.
83. Zhao, M.-D., Li, J.-Q., Chen, F.-Y., Dong, W., Wen, L.-J., Fei, W.-D., Zhang, X., Yang, P.-L., Zhang, X.-M., and Zheng, C.-H., *Co-Delivery of Curcumin and Paclitaxel by "Core-Shell" Targeting Amphiphilic Copolymer to Reverse Resistance in the Treatment of Ovarian Cancer.* International Journal of Nanomedicine, 2019. **Volume 14**: p. 9453-9467.
84. Wang, H.-X., Zuo, Z.-Q., Du, J.-Z., Wang, Y.-C., Sun, R., Cao, Z.-T., Ye, X.-D., Wang, J.-L., Leong, K.W., and Wang, J., *Surface charge critically affects tumor penetration and therapeutic efficacy of cancer nanomedicines.* 2016. **11**(2): p. 133-144.
85. Thurston, G., McLean, J.W., Rizen, M., Baluk, P., Haskell, A., Murphy, T.J., Hanahan, D., and McDonald, D.M., *Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice.* Journal of Clinical Investigation, 1998. **101**(7): p. 1401-1413.
86. Philipp, A., Meyer, M., and Wagner, E., *Extracellular Targeting of Synthetic Therapeutic Nucleic Acid Formulations* Current Gene

Therapy, 2008. **8**(5): p. 324-334.
87. Brannon-Peppas, L. and Blanchette, J.O., *Nanoparticle and targeted systems for cancer therapy.* Advanced drug delivery reviews, 2004. **56**(11): p. 1649-1659.
88. De Bono, J.S. and Ashworth, A., *Translating cancer research into targeted therapeutics.* Nature, 2010. **467**(7315): p. 543-549.
89. Šelemetjev, S., Bartolome, A., Išić Denčić, T., Đorić, I., Paunović, I., Tatić, S., and Cvejić, D., *Overexpression of epidermal growth factor receptor and its downstream effector, focal adhesion kinase, correlates with papillary thyroid carcinoma progression.* International Journal of Experimental Pathology, 2018. **99**(2): p. 87-94.
90. Lai, E.W., Joshi, B.H., Martiniova, L., Dogra, R., Fujisawa, T., Leland, P., De Krijger, R.R., Lubensky, I.A., Elkahloun, A.G., Morris, J.C., Puri, R.K., and Pacak, K., *Overexpression of Interleukin-13 Receptor-α2 in Neuroendocrine Malignant Pheochromocytoma: A Novel Target for Receptor Directed Anti-Cancer Therapy.* 2009. **94**(8): p. 2952-2957.
91. McFall, T., McKnight, B., Rosati, R., Kim, S., Huang, Y., Viola-Villegas, N., and Ratnam, M., *Progesterone receptor A promotes invasiveness and metastasis of luminal breast cancer by suppressing regulation of critical microRNAs by estrogen.* Journal of Biological Chemistry, 2018. **293**(4): p. 1163-1177.
92. Sudimack, J. and Lee, R.J., *Targeted drug delivery via the folate receptor.* Advanced Drug Delivery Reviews, 2000. **41**(2): p. 147-162.
93. Assaraf, Y.G., Leamon, C.P., and Reddy, J.A., *The folate receptor as a rational therapeutic target for personalized cancer treatment.* Drug Resistance Updates, 2014. **17**(4-6): p. 89-95.
94. Lu, Y. and Low, P.S., *Folate-mediated delivery of macromolecular anticancer therapeutic agents.* Advanced Drug Delivery Reviews, 2002. **54**(5): p. 675-693.
95. Lee, R.J. and Low, P.S., *Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1995. **1233**(2): p. 134-144.
96. Selvaggi, G., Novello, S., Torri, V., Leonardo, E., De Giuli, P., Borasio, P., Mossetti, C., Ardissone, F., Lausi, P., and Scagliotti, G.V., *Epidermal growth factor receptor overexpression correlates with a poor prognosis in completely resected non-small-cell lung cancer.* Annals of Oncology, 2004. **15**(1): p. 28-32.
97. Schrevel, M., Gorter, A., Kolkman-Uljee, S.M., Trimbos, J.B.M.Z., Fleuren, G.J., and Jordanova, E.S., *Molecular mechanisms of epidermal growth factor receptor overexpression in patients with cervical cancer.* 2011. **24**(5): p. 720-728.
98. Qian, Z.M., Li, H., Sun, H., and Ho, K., *Targeted Drug Delivery via the Transferrin Receptor-Mediated Endocytosis Pathway.* Pharmacol Rev., 2002. **54**(4): p. 561-87
99. Tanaka, T., Fujishima, Y., and Kaneo, Y., *Receptor Mediated Endocytosis and Cytotoxicity of Transferrin-Mitomycin C Conjugate in the HepG2 Cell and Primary Cultured Rat Hepatocyte.* Biological & Pharmaceutical Bulletin, 2001. **24**(3): p. 268-273.
100. Niitsu, Y., Kohgo, Y., Nishisato, T., Kondo, H., Kato, J., Urushizaki, Y., and Urushizaki, I., *Transferrin receptors in human cancerous tissues.* The Tohoku Journal of Experimental Medicine, 1987. **153**(3):

p. 239-243.
101. Martin, I., Dohmen, C., Mas-Moruno, C., Troiber, C., Kos, P., Schaffert, D., Lächelt, U., Teixidó, M., Günther, M., Kessler, H., Giralt, E., and Wagner, E., *Solid-phase-assisted synthesis of targeting peptide–PEG–oligo(ethane amino)amides for receptor-mediated gene delivery.* 2012. **10**(16): p. 3258.
102. Wang, L., Su, W., Liu, Z., Zhou, M., Chen, S., Chen, Y., Lu, D., Liu, Y., Fan, Y., Zheng, Y., Han, Z., Kong, D., Wu, J.C., Xiang, R., and Li, Z., *CD44 antibody-targeted liposomal nanoparticles for molecular imaging and therapy of hepatocellular carcinoma.* Biomaterials, 2012. **33**(20): p. 5107-5114.
103. Yang, J., Hirata, T., Croce, K., Merrill-Skoloff, G., Tchernychev, B., Williams, E., Flaumenhaft, R., Furie, B.C., and Furie, B., *Targeted Gene Disruption Demonstrates That P-Selectin Glycoprotein Ligand 1 (Psgl-1) Is Required for P-Selectin–Mediated but Not E-Selectin–Mediated Neutrophil Rolling and Migration.* The Journal of Experimental Medicine, 1999. **190**(12): p. 1769-1782.
104. Bi, Y., Hao, F., Yan, G., Teng, L., Lee, R.J., and Xie, J., *Actively Targeted Nanoparticles for Drug Delivery to Tumor.* Current drug metabolism, 2016. **17**(8): p. 763-782.
105. Zhao, R., Diop-Bove, N., Visentin, M., and Goldman, I.D., *Mechanisms of Membrane Transport of Folates into Cells and Across Epithelia.* Annual Review of Nutrition, 2011. **31**(1): p. 177-201.
106. Truebenbach, I., Gorges, J., Kuhn, J., Kern, S., Baratti, E., Kazmaier, U., Wagner, E., and Lächelt, U., *Sequence-Defined Oligoamide Drug Conjugates of Pretubulysin and Methotrexate for Folate Receptor Targeted Cancer Therapy.* Macromolecular Bioscience, 2017. **17**(10): p. 1600520.
107. He, D., Müller, K., Krhac Levacic, A., Kos, P., Lächelt, U., and Wagner, E., *Combinatorial Optimization of Sequence-Defined Oligo(ethanamino)amides for Folate Receptor-Targeted pDNA and siRNA Delivery.* Bioconjugate Chemistry, 2016. **27**(3): p. 647-659.
108. Lee, D.-J., Kessel, E., Lehto, T., Liu, X., Yoshinaga, N., Padari, K., Chen, Y.-C., Kempter, S., Uchida, S., Rädler, J.O., Pooga, M., Sheu, M.-T., Kataoka, K., and Wagner, E., *Systemic Delivery of Folate-PEG siRNA Lipopolyplexes with Enhanced Intracellular Stability for In Vivo Gene Silencing in Leukemia.* Bioconjugate Chemistry, 2017. **28**(9): p. 2393-2409.
109. Lee, D.-J., Kessel, E., Edinger, D., He, D., Klein, P.M., Voith Von Voithenberg, L., Lamb, D.C., Lächelt, U., Lehto, T., and Wagner, E., *Dual antitumoral potency of EG5 siRNA nanoplexes armed with cytotoxic bifunctional glutamyl-methotrexate targeting ligand.* Biomaterials, 2016. **77**: p. 98-110.
110. Truebenbach, I., Kern, S., Loy, D.M., Höhn, M., Gorges, J., Kazmaier, U., and Wagner, E., *Combination Chemotherapy of L1210 Tumors in Mice with Pretubulysin and Methotrexate Lipo-Oligomer Nanoparticles.* Molecular Pharmaceutics, 2019. **16**(6): p. 2405-2417.
111. Klein, P.M., Kern, S., Lee, D.-J., Schmaus, J., Höhn, M., Gorges, J., Kazmaier, U., and Wagner, E., *Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing in vivo.* Biomaterials, 2018. **178**: p. 630-642.
112. Zhang, W., Rödl, W., He, D., Döblinger, M., Lächelt, U., and Wagner,

E., *Combination of sequence-defined oligoaminoamides with transferrin-polycation conjugates for receptor-targeted gene delivery.* The Journal of Gene Medicine, 2015. **17**(8-9): p. 161-172.
113. Urbiola, K., Blanco-Fernández, L., Ogris, M., Rödl, W., Wagner, E., and Tros De Ilarduya, C., *Novel PAMAM-PEG-Peptide Conjugates for siRNA Delivery Targeted to the Transferrin and Epidermal Growth Factor Receptors.* Journal of Personalized Medicine, 2018. **8**(1): p. 4.
114. Salomon, D.S., Brandt, R., Ciardiello, F., and Normanno, N., *Epidermal growth factor-related peptides and their receptors in human malignancies.* Critical Reviews in Oncology/Hematology, 1995. **19**(3): p. 183-232.
115. Tomas, A., Futter, C.E., and Eden, E.R., *EGF receptor trafficking: consequences for signaling and cancer.* Trends in Cell Biology, 2014. **24**(1): p. 26-34.
116. Liu, X., Wang, P., Zhang, C., and Ma, Z., *Epidermal growth factor receptor (EGFR): A rising star in the era of precision medicine of lung cancer.* Oncotarget, 2017. **8**(30).
117. Morys, S., Urnauer, S., Spitzweg, C., and Wagner, E., *EGFR Targeting and Shielding of pDNA Lipopolyplexes via Bivalent Attachment of a Sequence-Defined PEG Agent.* Macromolecular Bioscience, 2018. **18**(1): p. 1700203.
118. Truebenbach, I., Zhang, W., Wang, Y., Kern, S., Höhn, M., Reinhard, S., Gorges, J., Kazmaier, U., and Wagner, E., *Co-delivery of pretubulysin and siEG5 to EGFR overexpressing carcinoma cells.* International Journal of Pharmaceutics, 2019. **569**: p. 118570.
119. Schäfer, A., Pahnke, A., Schaffert, D., Van Weerden, W.M., De Ridder, C.M.A., Rödl, W., Vetter, A., Spitzweg, C., Kraaij, R., Wagner, E., and Ogris, M., *Disconnecting the Yin and Yang Relation of Epidermal Growth Factor Receptor (EGFR)-Mediated Delivery: A Fully Synthetic, EGFR-Targeted Gene Transfer System Avoiding Receptor Activation.* 2011. **22**(12): p. 1463-1473.
120. Peterson, L.F., Wang, Y., Lo, M.C., Yan, M., Kanbe, E., and Zhang, D.E., *The multi-functional cellular adhesion molecule CD44 is regulated by the 8;21 chromosomal translocation.* Leukemia, 2007. **21**(9): p. 2010-2019.
121. Koukourakis, M.I., Giatromanolaki, A., Tsakmaki, V., Danielidis, V., and Sivridis, E., *Cancer stem cell phenotype relates to radio-chemotherapy outcome in locally advanced squamous cell head–neck cancer.* 2012. **106**(5): p. 846-853.
122. Misra, S., Heldin, P., Hascall, V.C., Karamanos, N.K., Skandalis, S.S., Markwald, R.R., and Ghatak, S., *Hyaluronan-CD44 interactions as potential targets for cancer therapy.* FEBS Journal, 2011. **278**(9): p. 1429-1443.
123. Resnick, N., Clarke, M., Siegfried, J., Landreneau, R., Asman, D., Ge, L., Kierstead, L., Dougherty, G., and Cooper, D., *Expression of the Cell Adhesion Molecule CD44 in Human Lung Tumors and Cell Lines* Mol Diagn, 1998. **3**(2): p. 93-103.
124. Wei, H.-J., Yin, T., Zhu, Z., Shi, P.-F., Tian, Y., and Wang, C.-Y., *Expression of CD44, CD24 and ESA in Pancreatic Adenocarcinoma Cell Lines Varies With Local Microenvironment* Hepatobiliary Pancreat Dis Int, 2011. **10**(4): p. 428-32.

125. Hiscox, S., Baruah, B., Smith, C., Bellerby, R., Goddard, L., Jordan, N., Poghosyan, Z., Nicholson, R.I., Barrett-Lee, P., and Gee, J., *Overexpression of CD44 accompanies acquired tamoxifen resistance in MCF7 cells and augments their sensitivity to the stromal factors, heregulin and hyaluronan.* BMC Cancer, 2012. **12**(1): p. 458.
126. Schanté, C.E., Zuber, G., Herlin, C., and Vandamme, T.F., *Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications* Carbohydrate Polymers, 2011. **85**(3): p. 469-489.
127. Bassi, P.F., Volpe, A., D'Agostino, D., Palermo, G., Renier, D., Franchini, S., Rosato, A., and Racioppi, M., *Paclitaxel-hyaluronic Acid for Intravesical Therapy of Bacillus Calmette-Guérin Refractory Carcinoma in Situ of the Bladder: Results of a Phase I Study* J Urol, 2011. **185**(2): p. 445-9.
128. Colnot, D.R., Quak, J.J., Roos, J.C., van Lingen, A., Wilhelm, A.J., van Kamp, G.J., Huijgens, P.C., Snow, G.B., and van Dongen, G.A., *Phase I therapy study of 186Re-labeled chimeric monoclonal antibody U36 in patients with squamous cell carcinoma of the head and neck.* Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 2000. **41**(12): p. 1999-2010.
129. Börjesson, P.K.E., Postema, E.J., Roos, J.C., Colnot, D.R., Marres, H.A.M., van Schie, M.H., Stehle, G., de Bree, R., Snow, G.B., Oyen, W.J.G., and van Dongen, G.A.M.S., *Phase I therapy study with (186)Re-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with head and neck squamous cell carcinoma.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2003. **9**(10 Pt 2): p. 3961S-72S.
130. Bundesministerium der Justiz und für Verbraucherschutz. *Tierschutzgesetz (TierSchG)*. 2019 [cited 2020 05 january]; Available from: https://www.gesetze-im-internet.de/tierschg.
131. Xu, X.-M., Chen, Y., Chen, J., Yang, S., Gao, F., Underhill, C.B., Creswell, K., and Zhang, L., *A peptide with three hyaluronan binding motifs inhibits tumor growth and induces apoptosis.* Cancer Res, 2003. **63**(18): p. 5685-90.
132. Robertson, R.T., Levine, S.T., Haynes, S.M., Gutierrez, P., Baratta, J.L., Tan, Z., and Longmuir, K.J., *Use of labeled tomato lectin for imaging vasculature structures.* Histochemistry and Cell Biology, 2015. **143**(2): p. 225-234.
133. Janvier. *NMRI-nu Immundeficient Mouse*. [cited 2020 10 february]; Available from: https://www.janvier-labs.com/en/fiche_produit/nmri-nu_mouse/.
134. Mann, B.J. and Wadsworth, P., *Kinesin-5 Regulation and Function in Mitosis.* Trends in Cell Biology, 2019. **29**(1): p. 66-79.
135. Lee, K., Jang, B., Lee, Y.-R., Suh, E.-Y., Yoo, J.-S., Lee, M.-J., Lee, J.-Y., and Lee, H., *The cutting-edge technologies of siRNA delivery and their application in clinical trials.* Archives of pharmacal research, 2018. **41**(9): p. 867-874.
136. Singh, A., Trivedi, P., and Jain, N.K., *Advances in siRNA delivery in cancer therapy.* Artificial Cells, Nanomedicine, and Biotechnology, 2018. **46**(2): p. 274-283.
137. Alagia, A. and Eritja, R., *siRNA and RNAi optimization.* Wiley

interdisciplinary reviews. RNA, 2016. **7**(3): p. 316-329.
138. Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R.K., Racie, T., Rajeev, K.G., Röhl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., and Vornlocher, H.-P., *Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs.* Nature, 2004. **432**(7014): p. 173-178.
139. Juliano, R., Bauman, J., Kang, H., and Ming, X., *Biological barriers to therapy with antisense and siRNA oligonucleotides.* Molecular pharmaceutics, 2009. **6**(3): p. 686-695.
140. Klein, P.M., Reinhard, S., Lee, D.-J., Müller, K., Ponader, D., Hartmann, L., and Wagner, E., *Precise redox-sensitive cleavage sites for improved bioactivity of siRNA lipopolyplexes.* Nanoscale, 2016. **8**(42): p. 18098-18104.
141. Müller, K., Klein, P.M., Heissig, P., Roidl, A., and Wagner, E., *EGF receptor targeted lipo-oligocation polyplexes for antitumoral siRNA and miRNA delivery.* Nanotechnology, 2016. **27**(46): p. 464001-464001.
142. Zhang, W., Müller, K., Kessel, E., Reinhard, S., He, D., Klein, P.M., Höhn, M., Rödl, W., Kempter, S., and Wagner, E., *Targeted siRNA Delivery Using a Lipo-Oligoaminoamide Nanocore with an Influenza Peptide and Transferrin Shell.* Advanced healthcare materials, 2016. **5**(12): p. 1493-1504.
143. Chang, P.V., Prescher, J.A., Sletten, E.M., Baskin, J.M., Miller, I.A., Agard, N.J., Lo, A., and Bertozzi, C.R., *Copper-free click chemistry in living animals.* Proceedings of the National Academy of Sciences, 2010. **107**(5): p. 1821-1826.
144. Dellian, M., Yuan, F., Trubetskoy, V.S., Torchilin, V.P., and Jain, R.K., *Vascular permeability in a human tumour xenograft: molecular charge dependence.* British Journal of Cancer, 2000. **82**(9): p. 1513-1518.
145. Hoshyar, N., Gray, S., Han, H., and Bao, G., *The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction.* Nanomedicine (London, England), 2016. **11**(6): p. 673-692.
146. Bazak, R., Houri, M., Achy, S.E., Hussein, W., and Refaat, T., *Passive targeting of nanoparticles to cancer: A comprehensive review of the literature.* Molecular and clinical oncology, 2014. **2**(6): p. 904-908.
147. Mattheolabakis, G., Milane, L., Singh, A., and Amiji, M.M., *Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine.* Journal of Drug Targeting, 2015. **23**(7-8): p. 605-618.
148. Valentine, M.T., Fordyce, P.M., and Block, S.M., *Eg5 steps it up!* Cell Division, 2006. **1**(1): p. 31.
149. Oberleithner, H., Wälte, M., and Kusche-Vihrog, K., *Sodium renders endothelial cells sticky for red blood cells.* Frontiers in Physiology, 2015. **6**.
150. Janas, M.M., Jiang, Y., Duncan, R.G., Hayes, A.N., Liu, J., Kasperkovitz, P.V., Placke, M.E., and Barros, S.A., *Exposure to siRNA-GalNAc Conjugates in Systems of the Standard Test Battery*

for Genotoxicity. Nucleic acid therapeutics, 2016. **26**(6): p. 363-371.
151. Zhang, L., Conejo-Garcia, J.R., Katsaros, D., Gimotty, P.A., Massobrio, M., Regnani, G., Makrigiannakis, A., Gray, H., Schlienger, K., Liebman, M.N., Rubin, S.C., and Coukos, G., *Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.* The New England journal of medicine, 2003. **348**(3): p. 203-213.
152. Giraldo, N.A., Becht, E., Remark, R., Damotte, D., Sautès-Fridman, C., and Fridman, W.H., *The immune contexture of primary and metastatic human tumours.* Current opinion in immunology, 2014. **27**: p. 8-15.

VIII. APPENDIX

1. Publications

Klein, P.M., Kern, S., Lee, D.J., Schmaus, J., Höhn, M., Gorges, J., Kazmaier, U., Wagner, E. **Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing *in vivo***. Biomaterials 2018, 178, 630-642.

Luo, J., Schmaus, J., Cui, M., Hörterer, E., Höhn, M., Däther, M., Hager, S., Benli-Hoppe, T., Peng, L., Wagner, E. **Hyaluronate siRNA nanoparticles with positive charge attach to tumor endothelium and penetrate into tumors**. Manuscript submitted for publication.

2. Posters

Luo, J., Schmaus, J., Cui, M., Hörterer, E., Wagner, E. **Surface charge influences the nanoparticle penetration into tumor.** CRS German Chapter Annual Meeting 2020: “Delivery and Formulation of Biologics”, Munich, Germany, February 20

IX. ACKNOWLEDGEMENTS

Zuerst möchte ich mich bei Prof. Dr. Ernst Wagner bedanken, der mir die Möglichkeit gegeben hat diese Dissertation an seinem Lehrstuhl anzufertigen. Seine Ratschläge und wissenschaftliche Expertise sowie das entgegengebrachte Vertrauen waren eine große Hilfe für die Anfertigung der Doktorarbeit.

Ich möchte mich ebenfalls bei Prof. Dr. Eckhard Wolf für die Übernahme meiner Betreuung an der tierärztlichen Fakultät bedanken.

Für die gute Zusammenarbeit möchte ich mich bei Simone Hager bedanken – genauso bei Elisa Hörterer, die nach kurzer Einarbeitung den Tierstall quasi im Alleingang (mit mir als Schreibkraft) geschmissen hat.

Thanks to Jie Luo for the great cooperation in the numerous *in vivo* experiments we performed together. I also have to thank Mochen Cui for his help concerning perfusion!

Natürlich gilt ein großer Dank auch Dr. Sarah Kern, die mich während des ersten Jahres meiner Doktorarbeit exzellent betreut hat und in dieser Zeit zu einer guten Freundin geworden ist.

Des Weiteren möchte ich mich bei unserem Tierpfleger Markus Kovac für die Versorgung der Tiere und bei Wolfgang Rödl für die unkomplizierte Hilfe bei jeglichem technischen Problem bedanken.

Ich möchte mich außerdem beim gesamten Arbeitskreis für die tolle Atmosphäre bedanken, sowohl während der Arbeit aber auch und v.a. bei den Events, die mit Arbeit wenig oder garnix zu tun hatten – Skifahren, Wiesn, Doktorfeiern, Geburtstage etc. etc!

Zwei langjährige Wegbegleiter und Freunde aus Schulzeiten (ich sag nur: PGA2010) dürfen an dieser Stelle natürlich nicht fehlen: Alex und Lange - danke für die gute Zeit bei jedem (feuchtfröhlichen) Aufeinandertreffen (ob in der fränkischen Heimat aufm Pfetzendorfer Festival oder im bayerischen bzw. schwäbischen Exil) und die Hilfe bei diversen Umzügen. Ich hoffe, dass wir auch in Zukunft noch das eine oder andere Bier(käst)chen zusammen vernichten!

Ein spezieller Dank geht an meine Eltern und meinen Bruder, auf deren Unterstützung ich auf meinem Lebensweg immer zählen konnte.

Zu guter Letzt möchte ich mich bei meiner Frau Christina bedanken: du lenkst meinen Blick auf die wirklich wesentlichen Dinge und sorgst dafür, dass ich auch an meinen schlechten Tagen schätzen kann, wie viel Glück ich in meinem Leben habe – ich freue mich auf unsere gemeinsame Zukunft!