Dendritic Polyglycerol for Efficient Drug and siRNA Delivery

Inaugural-Dissertation
to obtain the academic degree
Doctor rerum naturalium (Dr. rer. nat.)
submitted to the
Department of Biology, Chemistry, and Pharmacy of
Freie Universität Berlin

by

Fatemeh Sheikhi Mehrabadi

from Iran (Arak)

June, 2015

The following Ph.D. thesis was carried out within the research group of Prof. Dr. Rainer Haag from May, 2010 until June, 2015 at the Department of Biology, Chemistry, and Pharmacy of the Freie Universität Berlin.

I hereby declare that this Ph.D. thesis was prepared autonomously and that no illegal help was used. Contributions of others, e.g., content, quotes, or figures are indicated by referring to the original work.

Berlin, June 2015 _____

1. Reviewer: Prof. Dr. Rainer Haag
2. Reviewer: Jun.- Prof. Dr. Marcelo Calderón

Day of defense: 22.07.15

My Family

Acknowledgments

In the first place, I'd like to thank my Ph.D. advisor *Prof. Dr. Rainer Haag* for offering me the opportunity to be a member of his research group and for his support that helped me to continue my study and be a "mother" at the same time. I'm also grateful to him for his academic support during the course of this work. I'd like to thank *Jun.- Prof. Dr. Marcelo Calderón* for being the second referee of this work. I'm very much thankful to him for his scientific and nonscientific support over the last years.

Furthermore, I would like to acknowledge the whole members of *AG Haag* for the collaborative atmosphere and support. Particularly, I would like to thank my lab and office mates for friendly and collaborative atmosphere. Special thanks goes to *Jutta Hass* for organizing the financial and administrative issues. *Gaby Hertel* is acknowledged for chemical ordering and logistics. I'm grateful to *Dr. Pamela Winchester* for proofreading of my manuscripts and this thesis. I'm very thankful to *Cathleen Schlesener* for her help and cooperativeness during my pregnancy. I'm grateful to *Dr. Wiebke Fischer* for being always there to help and support, particularly by professionally solving graphical issues. She is further acknowledged for the careful proofreading of this thesis. I thank all the members of *AG Calderón* for the friendly atmosphere, especially *Mazdak Asadian-Birjand* for our Deutsch-Persisch hours and solving almost all the issues concerning computer problems and "Deutsche Sprache." Furthermore, I'm grateful to *Dr. Stefanie Wedepohl* for performing some of the biological studies and for invaluable discussions. I thank kindly *Karolina Walker* and *Mazdak Asadian-Birjand* for their efforts to translate my thesis abstract to German.

I'd like to thank my collaboration partners at Roche: *Dr. U. Brinkmann* and *J. Adelmann*, at University of California: *Prof. Zhibin Guan, Dr. H. Zeng, and M. Johnson, Dr. W. Kemmner* from the Charité Campus at Berlin Buch (CBB), and *Prof. Sabrina Pricl* and her group at University of Trieste for their contributions to this work.

All the analytical facilities in the *Organic Chemistry Department of Freie Universität Berlin* are acknowledged for numerous NMR and Mass measurements. I would like to highly acknowledge the financial support of *SFB 765* and Bundesministerium für Bildung und Forschung (*BMBF*) during the course of this Ph.D. work.

I'm indebted to *my parents* who encouraged and supported me during all these years to pursue my studies. I want to thank sincerely my husband *"Mohsen"* for his never ending optimism, endless support and endurance, especially during the long journey of this thesis. I also thank my little son/sun "*Amin*" who was my main source of energy during the last two and half years.

Finally, I'd like to thank all the people whom I might have forgotten to name but who contributed to shaping up this work.

Contents

*The highlighted sections have been published in the following review: “Dendritic and lipid-based carriers for gene/siRNA delivery” F. Sheikhi Mehrabadi, W. Fischer, R. Haag, *Current Opinion in Solid State and Materials Science* **2013**, *16*, 310-322.

1 Introduction

1.1 General Aspects

1.1.1 "Polymer Therapeutics"

The advent of "macromolecules," resulting from the pioneering work of Hermann Staudinger, and the advances in biomedical science resulted in nano-sized polymer-based pharmaceuticals, generally described as "polymer therapeutics."[1-2] "Polymer therapeutics" is a broad term that encompasses polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymer-aptamer conjugates, polymeric micelles (with drugs covalently linked to them), and multicomponent non-viral systems (with covalent linkages) for nucleic acid delivery (Figure 1).[1, 3] Conventional delivery systems simply entrapped, solubilized, or controlled the release of the drugs, whereas for polymer therapeutics, the covalent linkage of therapeutic agent is essential. Polymer therapeutics is classified as a subclass in "nanomedicine." "Nanomedicine" is an area of nanotechnology that deals with the design and development of constructs in the nanoscale range (1 to 200 nm) with a range of applications in healthcare from prevention and understanding of patho-physiology to diagnosis and therapy of diseases.[4]

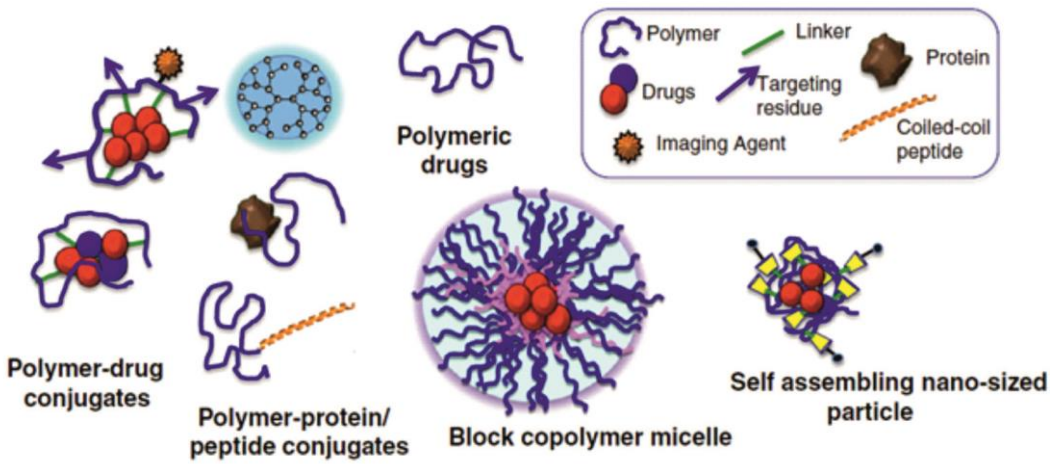


Figure 1. The schematic representation of different nanoscale structures employed as polymer therapeutics. Figure reprinted with permission from ref. [3]. Copyright Elsevier (2013).

Historically, the concept of using synthetic polymers as therapeutic agents goes back to the early 1940s.[3] The development of polymer-based materials in medicine started with the evaluation of polymers like dextran and poly(vinylpyrolidone) (PVP) (as plasma expanders), wound dressing materials, and disinfectants for clinical applications.[3, 5] Polymer-drug conjugates and polymer-protein conjugates (especially protein conjugates of polyethylene

glygol or PEG) started to appear in 1960.[3] Following intensive investigations, in the 1980s and 1990s, the first block copolymer micelles entered clinical trials.[6-8] Soon after that, in 1994, the first synthetic polymer-drug conjugate, N-(2-hydroxypropyl) methacrylamide (HPMA)-doxorubicin conjugates, reached clinical studies.[9] Currently a significant number of polymer therapeutics are in clinical developments or even entered the market.[4] Some examples of polymer therapeutics that have been marketed are: polymeric drugs like Copaxone®, polymer-protein conjugates like Zinostatin Stimaler® (marketed in Japan), PEGylated proteins like Cimzia®, Mircera®, and PEG-Interferon® and PEGylated-aptamers like Macugen®.

Today, polymer chemistry enables us to choose the appropriate carrier among a wide range of architectures including multifunctional linear, graft, star-like and dendritic polymers (Figure 2). The most repeatedly used polymeric structures particularly in the development of polymer-drug conjugates, are (N-(2-hydroxylpropyl) methacrylamide (HPMA) copolymer, poly-L-glutamic acid (PGA), PEG, and dextran.[9]

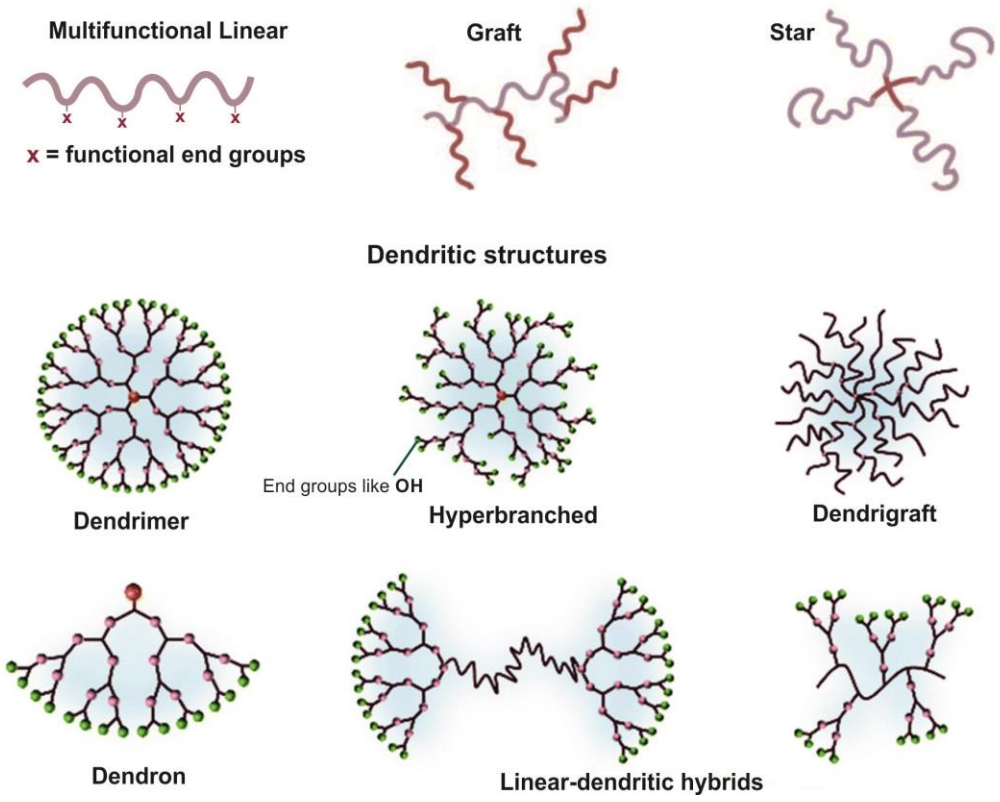


Figure 2. Examples of polymeric architectures for application in polymer therapeutic. Figure adapted with permission from ref. [1] and [10]. Copyright Nature Publishing Group (2003) and Royal Society of Chemistry (2015).

1.1.2 Endocytosis

One of the ultimate goals in nanomedicine is to bring the therapeutic agent into the specific site of action. There are different sites of action, depending on the drug type. While plasmid DNA (pDNA) should be delivered into the cell nucleus, the site of function for short interfering RNA (siRNA) is in cytosol. In fact, in most cases, endocytic pathways should be passed through to achieve a therapeutic effect. A great number of studies have focused on modulation of nanoscale structures with different material compositions, physical and chemical features to render cell entry.[11] After intravenous administration and by overcoming the extracellular barriers, drugs reach the plasma membrane. Plasma membrane is a complex and dynamic construct composed of a lipid bilayer that separates the intracellular milieu from the extracellular surrounding and plays a vital role in homeostasis.[12] As the surface of the cellular membrane is decorated with anionic glycosylated membrane proteins,[13] charged molecules, especially highly negative nucleic acids, have difficulty interacting with plasma membrane. Furthermore, hydrophobic molecules diffuse through the cell membrane into the cytosol. Small and essential molecules, such as sugars, amino acids, and ions, pass through plasma membrane protein pumps and channels. Large molecules, however, need to be transported through vesicles derived from cellular membrane. External entities like macromolecules and pathogens (like toxins or bacteria) or fluids, cross the plasma membranes through different pathways, which are generally termed endocytosis (Figure 3).[14]

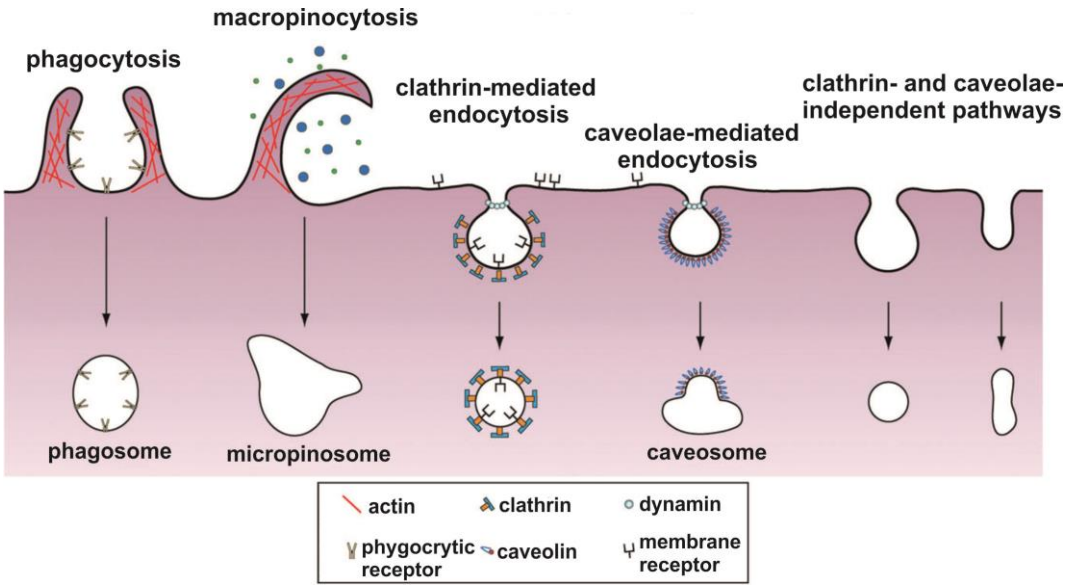


Figure 3. Different pathways of cell entry. Figure adapted with permission from ref. [11]. Copyright Royal Society of Chemistry (2011).

Endocytosis is broadly classified as phagocytosis[15-16] (cell eating) and pinocytosis (cell drinking). Large particles (1-10 µm), for example, aggregates of nanomaterials are internalized by phagocytosis. While phagocytosis is mainly a feature of phagocytic cells like monocytes and phagocytic and dendritic cells, pinocytosis is a universal uptake mechanism. Macrophages remove large pathogens and cell debris through phagocytosis. Pinocytosis occurs through at least four basic mechanisms; clathrin-mediated endocytosis (CME),[17] macropinocytosis, caveolae-mediated endocytosis,[18] and clathrin and caveola independent endocytosis.[19] During phagocytosis, large particles enter the cytosol by engulfing the cellular membrane into vesicular membranes termed phagosomes. In macropinocytosis, raffling of the plasma membrane results in internalization of a large volume of extracellular fluids. Caveolae are invaginations of the membrane and very abundant in endothelial cells. Clathrin-mediated endocytosis is among the most studied receptor mediated endocytosis, but many aspects in this endocytic pathway are still not understood. This pathway is highly complex and involves more than 50 proteins.[14] Briefly, in clathrin-mediated endocytosis, a coat of proteins is formed at the site of endocytosis that induces invagination of the membrane into spherical vesicles. These so-called clathrin pits are then cut and released into cytosol by enzyme dynamin.[20]

1.1.3 EPR Effect

Nano-sized polymer-based constructs can passively enter and be retained in tumor tissues after systemic administration. This happens because of the highly permeable characteristics of tumor vasculature, the porous endothelial layer, and poor lymphatic drainage of tumor tissue (Figure 4).

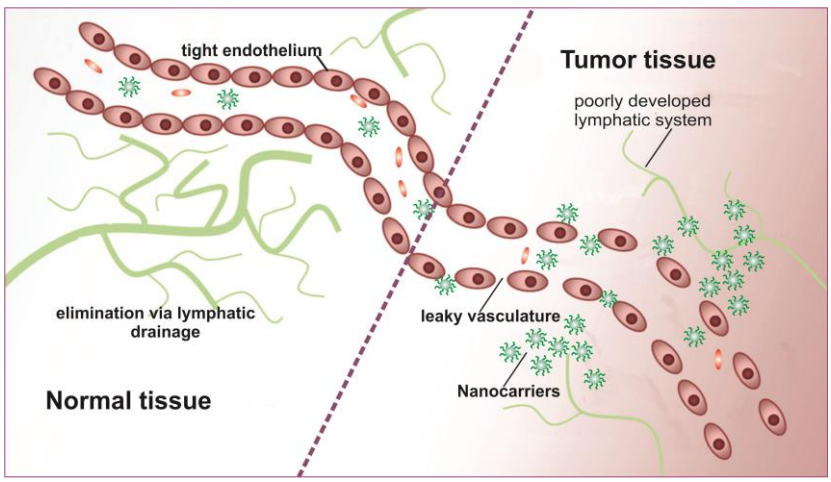


Figure 4. Enhanced permeation and retention (EPR) effect. Figure adapted with permission from ref. [21]. Copyright American Chemical Society (2012).

In addition to targeted delivery approaches based on ligand-receptor interactions, specific delivery of "nano-sized medicine" into a tumor site can be achieved by utilizing the effect known as the EPR (enhanced permeation and retention) effect.[22] With a vascular cut-off of about 500 nm, nano-sized carriers preferentially accumulate in the tumor tissues rather than normal tissues.[23] The reason for the high permeability of tumor tissue is an overexpression of the vascular permeability factor (VPF) and vascular endothelial growth factor (VEGF) and secretion of hormones like basic fibroblast growth factor (bFGF).[24] The EPR effect has been demonstrated in several studies using micelles,[7] liposomes,[25] polymer conjugates,[26-27] and nanoparticles.[28]

1.1.4 RNA Interference (RNAi), Promises and Pitfalls

The discovery of the catalytic properties of RNA in the early 1980s, post transcriptional gene silencing in plants,[29] proof of specific gene silencing in the nematode worm *C. elegans* in the 1990s,[30] sequence specific gene silencing using siRNA in mice,[31] and recent evidences of gene silencing in humans using siRNA[32] are all breakthroughs that expand the scope of RNA function beyond a passive intermediate between DNA and protein.

Among the recent advances, RNA interference (RNAi) has attracted a great deal of attention. This process is a regulatory mechanism that can control the expression of endogenous genes by base pairing of small RNAs (like siRNA) with messenger RNA (mRNA). This pathway first emerged as a result of probing the gene function using antisense approaches.[33] Soon after the awareness of the RNAi mechanism in mammalian cells in 2001,[34] and its role in gene expression control, it became clear that this process can be harnessed to study gene regulation and function in eukaryotes.[35-36] Particularly, the high specificity of gene silencing by RNAi could be utilized as a new class of drugs that are gene-specific and can silence diseases-causing or disease-promoting genes.[37-38]

Although early studies suggested that siRNA held the potential to selectively target and silence any gene, recent advances, however, suggest that harnessing this intrinsic potential in reality remains a great challenge. Nevertheless, siRNA based therapeutics are still considered an attractive strategy to address multiple diseases because they "*can be developed*" to silence any gene, have a picomolar range efficacy that can reduce possible adverse side effects, and can have a long lasting (a few weeks) therapeutic effect. Moreover, siRNA synthesis is relatively straightforward compared to therapeutic proteins. Nonetheless the significant

therapeutic potential of siRNA needs to be realized by addressing the problems of "*delivery, delivery, and delivery"*.[39]

1.1.5 Gene Silencing Mechanism by siRNA and miRNA

Gene regulation is mainly governed by two types of small RNA molecules with 20-30 nucleotides. These molecules are microRNA (miRNA) and siRNA. In spite of their fundamental similarities, miRNA and siRNA have different cellular origins and target recognition modes.[40] The origin of siRNA may be endogenous or exogenous, whereas miRNAs originate from genome. Another main difference between siRNA and miRNA is in their structural features. Double strands of RNA (dsRNA), as precursor of siRNA duplexes, has a perfect base pairing. miRNA helices in contrast, own mismatches and extended terminal loops.

Gene silencing by siRNA starts with the cleavage of long (~30 base pair) dsRNA in the cytoplasm by an endoribonuclease named Dicer. The resulting siRNA is then loaded into a complex called RISC. In RISC, the catalytic component Argonaute 2 (AGO2) cleaves and releases one strand of the RNA. As a result, RISC becomes activated and can specifically target mRNA with the help of a guide strand through complementary base pairing. The mRNA is then cleaved between base 10 and 11 relative to the 5' end of the siRNA guide strand, which causes mRNA degradation, prevents translation, and thereby silences the gene (Figure 5a).[41]

Long primary miRNA (Pri-miRNA) are transcribed from endogenous miRNA genes by RNA polymerase III. These primary transcripts contain stem-loop structures with 65-70 nucleotides. Later, pri-miRNA transcripts are cleaved by ribonuclease *Drosha*, an enzyme from RNase III-family in the nucleus to generate the precursor miRNA (or pre-miRNA). The resulting hairpin structures are transported into cytoplasm where they are incorporated into RISC. The following steps proceed with a similar mechanism like siRNA. Perfect complementary base pairing to the mRNA in the case of siRNA results in degradation of respective mRNA whereas partially complementary binding of miRNA to the mRNA cause mRNA translational arrest (Figure 5b).

The control over the gene expression by these two types of small RNAs generates a great opportunity to target many diseases. Today, it is known that miRNA dysregulations play a critical role in various diseases such as heart diseases, viral infections, and cancer.[42] Particularly, in cancer, miRNAs regulate tumorigenesis by either an upregulation of oncogenic

miRNAs or a downregulation of tumor-suppressing miRNAs. Therefore, therapeutic actions may be realized by either induction of RNAi as result of introducing synthetic pre-miRNA (or miRNA mimics) or inhibiting this pathway by blocking the RNA strand in the RNA induced silencing complex (RISC) (Figure 5b, pathway 1 or 2).[43]

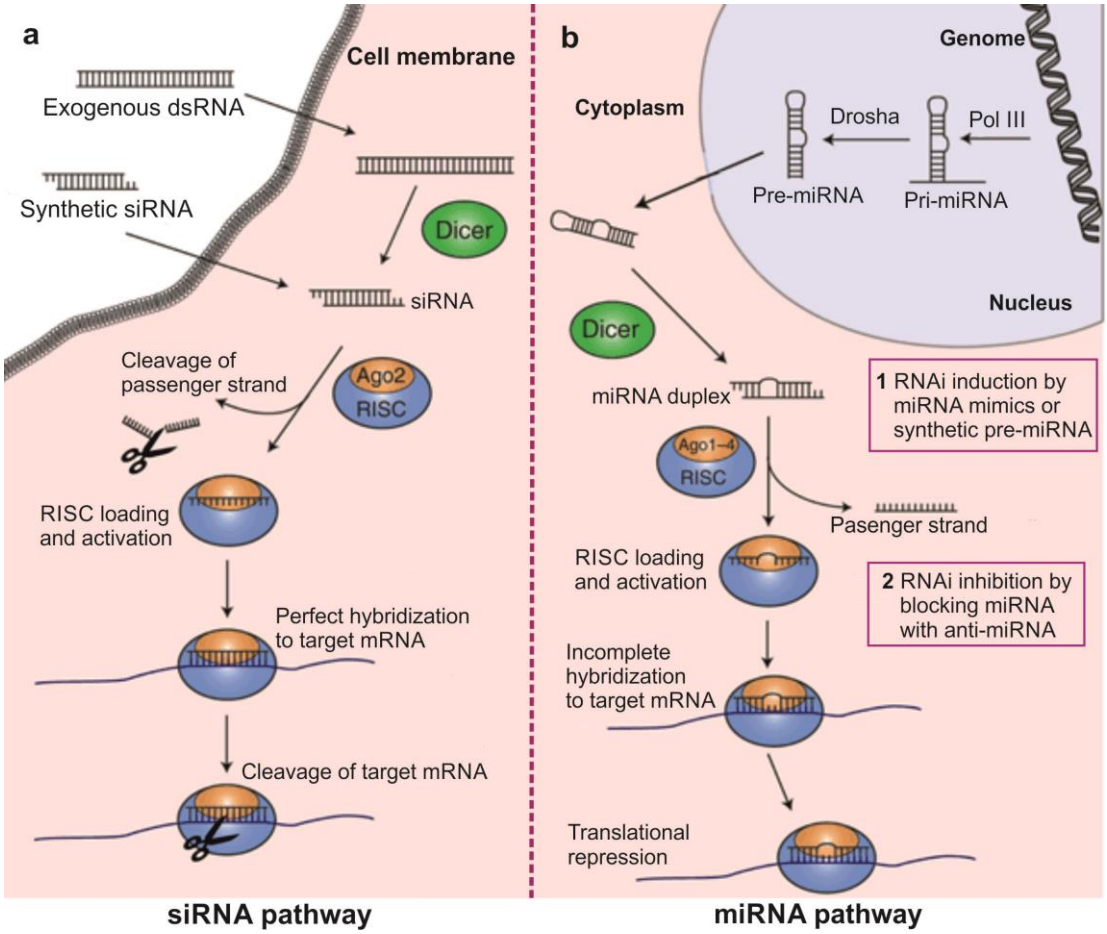


Figure 5. a) siRNA gene silencing pathway. Cleavage of dsRNA by Dicer followed by siRNA loading into the RISC. Cleavage and release of passenger strands of RNA by AGO2. Targeting mRNA by RNA guide strand, cleavage of mRNA, and resultant gene silencing. b) miRNA gene silencing pathway. Pri-miRNAs are cleaved by Drosha and form pre-miRNAs which are exported to the cytoplasm. The enzyme Dicer binds the pre-miRNA and cleaves the mature miRNA from hairpin structure. Passenger strand is released and guide strand with several proteins of RISC bind non-complementary to the mRNA resulting in translational arrest. 1 and 2 are possible pathways to induce or inhibit RNAi by miRNAs. Figure was reprinted with permission from ref. [44]. Copyright Nature Publishing Group (2012).

1.1.6 siRNA Modifications

siRNA molecules cannot readily enter the cells due to their macromolecular nature, high negative charge, and hydrophilicity.[45] To facilitate the uptake of siRNA molecules into the target site, siRNA may be chemically modified. Nonetheless, cellular entry is only one reason

to modify siRNA molecules. For *in vivo* applications, siRNA needs to avoid enzymatic cleavage that can trigger the innate immune systems as well.[46-48] 2´-O-methyl modification of the sugar moieties on certain nucleotides in both siRNA strands can reduce such activation of the immune system. In addition to 2´-O-methyl incorporation, 2´-methoxyethyl, 2´-deoxy (2´H), 2´-fluoro, locked nucleic acids (LNAs), phosphorothioate (PS) or boranophosphate derivatives (BP) have been shown to improve the enzymatic stability of siRNA therapeutics (Figure 6).[49-51]

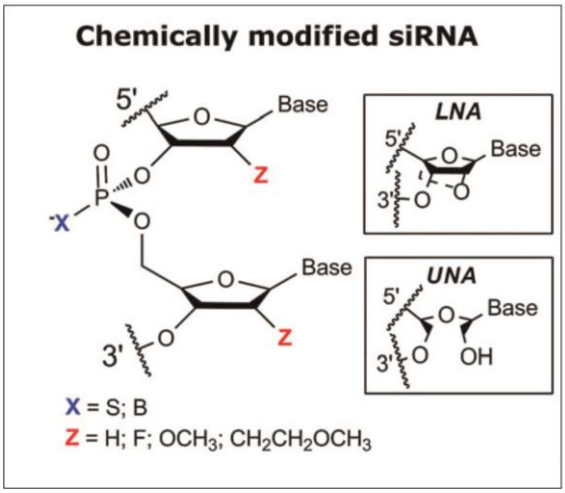


Figure 6. Schematic representation of various siRNA modifications. Chemical modifications (red and blue groups) or changes in the ribose conformation (LNA; UNA) render siRNA enzymatic stability. Figure has been adapted with permission from ref. [52]. Copyright Royal Society of Chemistry (2013).

Moreover, conjugation of siRNA to lipophilic groups like cholesterol or formation of other siRNA lipophilic conjugates[53-54] like Toc–siRNA and bile- and fatty acid–siRNA have been shown to improve their plasma protein binding, pharmacokinetics, and tissue distribution.[39] Furthermore, siRNA molecules have been modified to decrease the toxicity associated with non-selective gene silencing or the so called off-target effects. These modifications include incorporation of 2´OMe, 2´F, 2´H, and unlocked nucleic acids (UNA) on the siRNA guide strand, changes of the purine and pyrimidine bases, and altering the number of siRNA nucleotides in both siRNA strands.[52]

1.1.7 Direct or Systemic siRNA Delivery

The incorporation of siRNA into the RNAi machinery requires the delivery of this molecule into the cytoplasm of the cell. Delivery of the siRNA into cytoplasm depends on the targeted

organ and the route of administration. Systemic, local, and topical administrations are the three main modes of siRNA delivery.[45] In general, direct (local and topical) delivery of the siRNA into the target tissue encounters fewer barriers than systemic delivery. Higher bioavailability and lower risk of unfavourable effects are further advantages of direct delivery. The topical approach has been utilized for siRNA delivery into organs like eye,[55] skin,[56] and vagina[57] targeting macular degeneration, atopic dermatitis, and herpes simplex virus diseases, respectively. Examples of local siRNA delivery into organs include lung[58] in severe acute respiratory syndrome (SARS), brain[59] (Huntington's disease), and spinal cord[60] (chronic pain). In contrast to direct delivery, systemic administration of siRNA formulations (siRNA + delivery system) allows one to access a wider range of tissues for therapy. Organs like liver (hypercholesterolemia), heart (myocardial infarction), kidney (kidney disease), and metastasized tumors (Ewing's sarcoma) which cannot be targeted by direct routes become accessible for therapy upon systemic administration.[45] Further details about the siRNA systemic administration route and its hurdles are discussed in the next section.

1.1.8 *In vivo* Barriers of siRNA Formulations*

siRNA formulations, introduced through the systemic route, face multiple hurdles before reaching their site of action, cytosol.[45] Following intravenous administration of siRNA molecule and due to its relatively small size, rapid renal excretion happens with a plasma clearance rate (C_L) of 17.6 ml min^{-1}.[61] Particles with a hydrodynamic size of less than 10 nm cannot avoid renal clearance. Complexation with a carrier can increase the size of the particle and decrease the chance of kidney filtration. The next challenge starts as siRNA meets blood components as well as extracellular elements. siRNA formulations can build aggregates with serum proteins, which are removed by phagocytes (Figure 7a). Phagocytic cells like macrophages and monocytes efficiently remove any foreign material and are considered an important immunological barrier.[62] siRNA molecules are also highly susceptible to degradation by RNases (Figure 7b) both *ex* and *in vivo*. The stability of these molecules in the exposed environment is extremely important for an optimal clinical effect. siRNA encapsulation/complexation with an appropriate carrier may increase the stability of the siRNA molecules. Another obstacle in the delivery of the siRNA formulations to certain tissues is the extravasation from the blood stream. If the size of the molecule is larger than 5 nm, it cannot cross the endothelium and will stay in the blood till clearance. However, in some

reticuloendothelial system (RES) organs, such as liver and spleen as well as tumor tissue, molecules up to 200 nm are able to pass through (Figure 7c).[63]

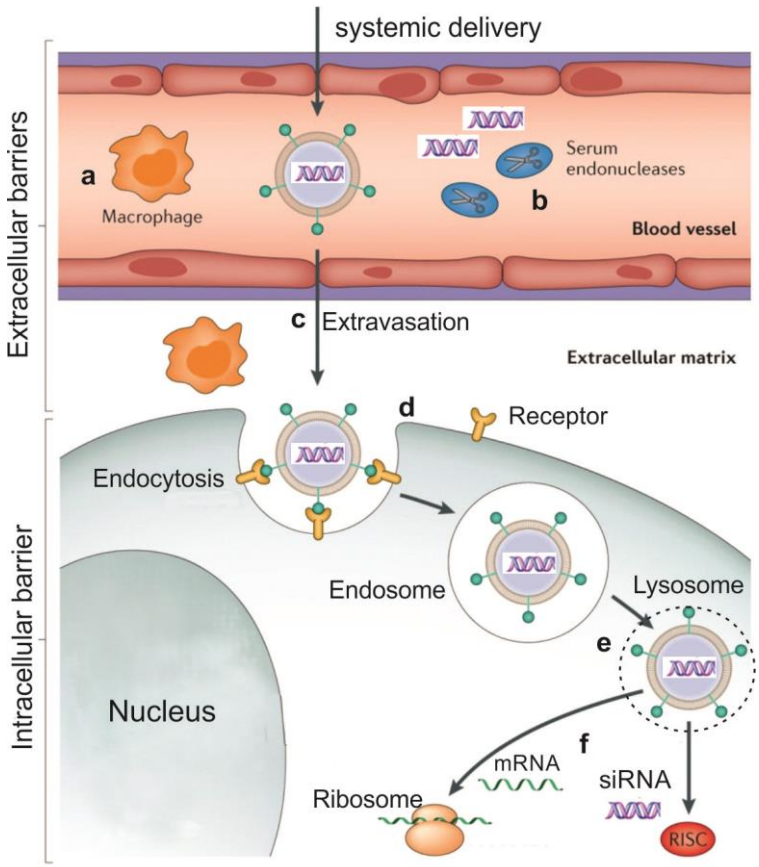


Figure 7. Challenges and barriers ahead of siRNA formulation after systemic delivery. a) Undesired interactions of siRNA formulations with blood components and phagocytes, b) degradation by RNA nucleases, c) extravasation from the blood stream, d) cellular membrane barrier, e) endosomal release, f) siRNA release form the carrier and accessibility to RNAi machinery. Figure adapted with permission from ref. [64]. Copyright Nature Publishing Group (2014).

After overcoming the blood endothelial wall barrier, the siRNA complex arrives in the vicinity of the target cell and has to pass through several physical barriers including cellular membrane (Figure 7d). Due to their relatively large size (~13 kDa), hydrophilicity, and highly negative charge, "naked" siRNA molecules cannot cross the biological membranes. However, complexation of the siRNA can promote its endocytosis, especially in the presence of binding ligands. In cytoplasm, siRNA formulation should escape from the endosomes before ending up in the lysosome where the pH significantly drops and degradative conditions can affect siRNA formulation and degrade the siRNA (Figure 7e). Ultimately, siRNA should be released from its carrier (Figure 7f) and become accessible to the RNAi machinery.[65]

1.2 Gene Delivery Systems

1.2.1 Viral Gene Delivery Systems, Benefits and Drawbacks

Gene therapy through different mechanisms, including introduction of a corrective gene to express a specific protein in transfected cells (gene augmentation), site specific gene repair,[66] and gene silencing approaches, can possibly treat or slow down the progression of many diseases.[67-68] Classical gene therapy has been mainly dominated by viral vectors that resulted from replacing the main part of viral genome with therapeutic nucleic acids. Viral vectors[69] are still considered the most efficient nucleic acid delivery systems.[70] In fact, viruses such as adenovirus,[71] adeno-associated virus (AAV),[72] and retrovirus[73] have learned sophisticated mechanisms over many years that surmount multiple barriers to deliver their genome into host cells efficiently. Generally, the results of viral vector applications for therapeutic purposes have been mixed. The catastrophic death of a patient due to excessive innate immune responses after a clinical trial of adenoviral gene therapy[74] or the development of leukemia following retroviral gene therapy[75] are on the dark side of their application history. On the other hand, there have been reports of successful viral vector applications.[76-77] Overall, the concerns about excessive immune responses upon utilization of viral vectors, their low cargo loading capacity, and wild-type virus reproduction remain the main road blocks for their broader application. Other difficulties like complexity of production, especially problems regarding large scale production, remain to be addressed.[78-79] Therefore, a great deal of effort has been devoted to modifying viral systems[80] to make them safer nucleic acid vehicles. Due to the limitations of viral vectors, approaches like designing “synthetic viruses” may be better alternative approaches.[81] Indeed a number of studies have focused on mimicking viral delivery systems using polymers, lipids,[82] and inorganic materials for nucleic acid delivery.[81, 83] Furthermore, other physical and mechanical methods like injection of naked siRNA/DNA, electroporation,[84] sonoporation,[85] microinjection[86] and microfluidic approaches[87] also have been explored as non-viral delivery strategies.

1.2.2 Non-Viral Gene Delivery Systems

1.2.2.1 Polymeric Nanocarriers

Non-viral gene delivery systems can be broadly classified into polymeric carriers,[88] lipid-based carriers,[89-90] dendrimer-based carriers,[88] peptides,[91] and other nanoparticles.[88]

Polymers and lipid-based materials are among the most repeatedly used non-viral vectors. Cationic polymers form polyelectrolyte complexes with negatively charged phosphate backbone of the nucleic acids called polyplexes. Cationic lipids with a hydrophobic tail and a positively charged head group, on the other hand, interact through their positively charged head groups with genetic materials to form supramolecular structures called lipoplexes.

Polymeric systems with different architectures, shapes, and molecular weights have been employed for siRNA delivery.[88] These polymeric materials can be broadly divided into two classes of natural and synthetic polymers. Chitosan and Cyclodextrin (CD) are well-studied examples of natural biopolymers that have found application in nucleic acid delivery.[92-93] The main reason for the application of natural polymers is their inherent biocompatibility and the toxicity issues associated with synthetic analogues. CD has found even more attention due to the successful application of a CD-based delivery system which is the first polymeric compound to enter clinical trials.[32]

1.2.2.1.1 Natural Polymeric Nanocarriers

Chitosan

Chitosan is a biocompatible, biodegradable, and positively charged linear polymer with low immunogenicity and cytotoxicity. Due to these characteristics, chitosan is considered an appropriate non-viral candidate for nucleic acid complexation and delivery.[94-96] Chitosan is a polysaccharide composed of D-glucosamine and *N*-acetyl-D-glucosamine units that are linked through a β-(1,4) glycosilic bond. Since more than two decades, many studies have focused on modulation of chitosan scaffold to gain higher transfection efficiency.[97] Investigations were done on many aspects, including the correlation between MW and nucleic acid delivery efficiency,[98] improved chitosan's cationic charge density,[99] buffering capacity,[100] and transfection efficiency.[101]

Cyclodextrin

The first report of using CD-based material for gene delivery was in 1999 for *in vitro* delivery of pDNA.[102] CDs are naturally occurring cyclic oligosaccharides composed of α-(1,4) coupled D-glucopyranose units. CD-based polymers (CDPs) were optimized by the group of Mark E. Davis for over a decade.[103] As a result of these intensive studies, a polymer-based delivery system was ready to enter clinical trials in 2008 (Figure 8).

The main component of this system was low molecular weight cyclodextrin polycationic oligomers (n ~ 5), an adamantine-PEG conjugate (AD-PEG), and adamantine-PEG transferrin conjugate (AD-PEG-Tf). CD oligomers contain a highly basic amidine functionality that guarantees an efficient condensation of nucleic acids at low N/P ratios. Introduction of imidazole groups at the termini of CD resulted in improved endosomal release properties.[104]

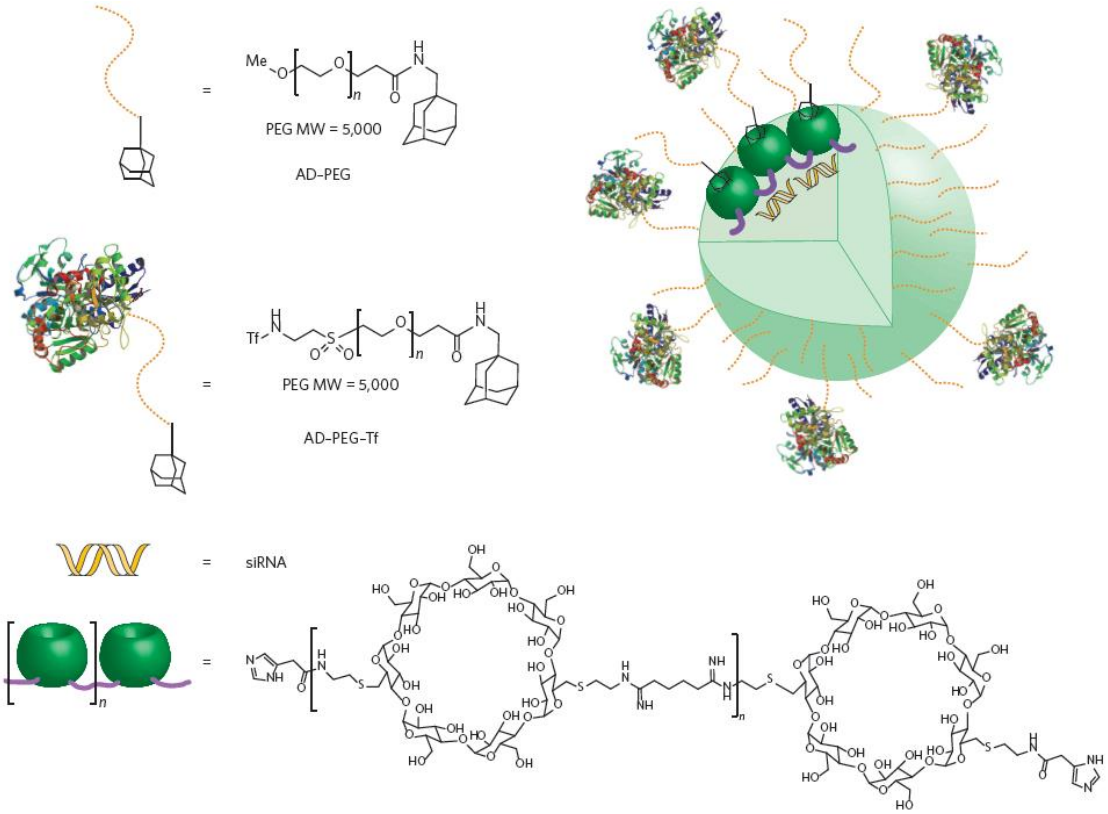


Figure 8. Schematic detailed structure of a CD-based polymeric nanocarrier that entered clinical trials (CALAA-01). Figure was reprinted with permission from ref. [105]. Copyright Nature Publishing Group (2013).

Adamantine-PEG (AD-PEG) and adamantine-PEG Transferrin (AD-PEG-Tf) were incorporated to CD to improve the properties of final nanoparticles for *in vivo* applications.[106] The surface of the CDP could be decorated by PEG shell[107] and Transferrin as a result of adamantine inclusion with cyclic part of CD to improve efficacy.[108] After evaluation of these systems in silencing several cancer associated genes,[109] the translation of targeted CDP-based system, CALAA-01, was examined in a dose-range study against ribonucleotide reductase subunit M2 (RRM2), in non-human primates.[110] Finally, the siRNA mediated cleavage of RRM2 mRNA using CALAA-01 was proven in phase I clinical trial on melanoma patients.[32]

1.2.2.1.2 Synthetic Polymeric Nanocarriers

Poly(ethylene imine) (PEI)*

Dendrimers as a new class of synthetic polymeric structures appeared in 1980s.[111] Compared to linear or traditional branched structures, dendrimers have various advantages including multifunctionality (for multivalent interaction and recognition in biological systems),[112] defined structures (a controllable dimension and composition), 3D architectures, and low dispersity. For dendrimers as an attractive class of macromolecules, many applications have been foreseen from solubility enhancers,[113-114] contrast agents in MRI,[115-117] gene and drug delivery platforms[118-121] to photodynamic therapy[122-125] and beyond.[126]

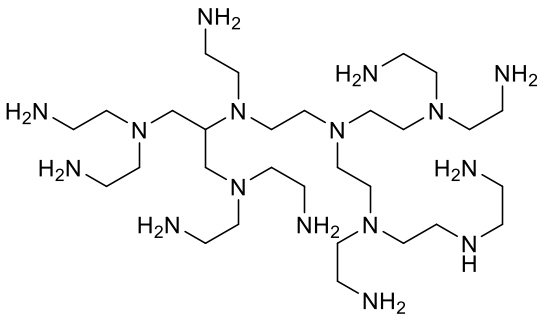


Figure 9. Hyperbranched poly(ethylene imine) (PEI), the depicted structure represents only a small idealized fragment of hyperbranched high MW PEI.

Hyperbranched poly(ethylene imine) (PEI) is a commercially available polymer, which has been produced for about 50 years on a ton scale by BASF (Lupasol®).[127] Today, PEI can be produced with a relatively high degree of branching (62-84%), narrow molecular weight distributions (polydispersity index (PDI) typically below 2.0),[127] and number average molecular weights (M_n) up to 10000 g/mol. This corresponds to the typically used PEI 25 kDa (with PDI of about 2.5) as well. Due to its high transfection efficiencies *in vitro*, PEI is often considered as the gold standard for gene delivery studies. A variety of PEI derivatives have already been tested and numerous physical characterizations of PEI/DNA complexes including size, shape, surface charge, and concentrations for their gene transfer efficiency have been performed. Most of them focused on both the degree of branching and the influence of molecular weight on transfection efficiency.

The first studies in this direction using PEI were reported by Behr et al. in 1995.[128] Later a systematic structural analysis was performed by Kissel et al. to elucidate the effects of molecular weight and degree of branching on the efficiency of PEI for intracellular delivery of the genes. In 1999 this group reported the synthesis of low molecular weight PEI (LMW-PEI)

and compared its transfection properties and cytotoxicity profile to commercially available high molecular weight PEI (800 kDa, HMW-PEI).[129] They observed that the LMW-PEI showed a lower degree of branching that had a relatively low *in vitro* cytotoxicity profile and 100-fold higher expression of luciferase in 3T3 cells than HMW-PEI. The authors postulated that the higher transfection efficacy of the low MW analogues was caused by the lower cytotoxicity and the formation of stable nano-sized particles capable of being endocytosed. Furthermore, they reported the synthesis of LMW-PEI with a degree of branching of 50%, which resulted in an improved gene vector in comparison to commercially available PEI of 25 kDa MW. The excellent properties are believed to be the result of both the lower molecular weight and reduced degree of branching.[130]

Furthermore the effect of grafting poly(ethylene glycol) (PEG) on PEI in DNA transfection and stability of final polyplexes was studied.[131] By analyzing the PEG length and density effect on the transfection efficiency *in vitro*, it was found that DNA most effectively transfected, when a high density of relatively short (550 Da) PEG chains were grafted onto PEI.[132] Interestingly, siRNA gene silencing was more effective when a low density of longer (5 kDa) PEG chains was conjugated to PEI. To overcome the high toxicity of PEI and the relatively low transfection efficiency of the pegylated PEI systems (PEG-PEI), several examples were reported using targeting moieties which promoted an enhanced cellular uptake.[133]

Melittin is an active peptide with membrane destabilizing properties. To tune the lytic activity of this peptide under acidic condition in endosome, several analogues of this peptide were prepared by substitution of a few neutral glutamines groups in its sequence with glutamic acid residues. The conjugation of new melittin analogues greatly improved the endosomal release properties of PEI (25 kDa). The enhanced endosomal release properties notably improved the gene transfer capacity of PEI in four various cell lines.[134] In a similar study, melittin, was used to improve the efficiency of siRNA transfection by poly L-lysine (PLL) and PEI. In order to switch the lytic activity of this peptide in the site of action and under acidification in lysosome, dimethylmaleic anhydride (DMMAn) was used to mask the amine groups of this peptide. A pH-responsive analogue of melittin peptide could be prepared this way. Furthermore, PLL and PEI were post-modified with hydrophilic PEG to achieve polyplexes with an appropriate size for transfection. The siRNA transfection efficacy of two polycations significantly increased as result of this functionalization.[135]

The application of cationic polymers, particularly for repeated *in vivo* administrations, is limited due to the high toxicity and lack of biodegradability associated with cationic polymeric carriers. Therefore, as an alternative approach, many laboratories have focused on biodegradable polymeric scaffolds. A successful example of such systems was introduced by Wagner and coworkers. A β-propionamide-cross-linked analogue of oligoethylenimine (OEI), OEI-HD, conjugated to serum protein transferrin (Tf), as targeting agent, was prepared and evaluated for siRNA transfection *in vitro* and *in vivo*. As result, an efficient knockdown of luciferase gene in neuroblastoma cells and a specific down regulation of Ran protein expression and induction of apoptosis in Neuro2A tumors in mice model were attained.[136]

Furthermore, Wagner and coworkers have utilized the solid phase peptide synthesis strategy to synthesize sequence defined Fmoc/Boc protected artificial oligo(ethylene amino) acids consisting of a different number of diaminoethane units (for endosomal buffering properties), lysines (as branching units), cysteine (for formation of bioreversible disulfide bonds), and a hydrophobic domain. Therefore, a library of defined i-, T-, and U-shape polymers were synthesized and compared for efficient pDNA and siRNA delivery. This study demonstrated the critical role of polyplex stabilization by cysteine residues, the number of diaminoethane units for efficient DNA/siRNA binding, and enhancement of membrane disruptive and endosomal release properties of the final carrier by introducing protonable amines (at low endosomal pHs) and hydrophobic residues like linolic acid.[137]

Poly(amidoamine) (PAMAM)*

Poly(amidoamine) (PAMAM) dendrimers were first introduced by Tomalia and coworkers.[138] These dendrimers consist of repeating amide and amine units (Figure 10). They are synthesized by repeating the Michael addition of an amine into an α,β-unsaturated ester followed by amidation with excess of ethylene diamine.

In an important study, transfection by PAMAM was reported for the first time by Haensler and Szoka in 1993.[139] After that, Kukowska-Latallo et al. explored transfection efficiency of different generations [G5-G10] of PAMAM dendrimer in mammalian cells.[140] Stability of the DNA-PAMAM complexes towards nuclease degradation was also explored by the same group.[141] Later on in a key study, the role of proton sponge effect in transgene delivery was investigated with cationic polymers containing buffering amines.[142] Buffering occurs when

the low pH within the endosome promotes the uptake and accumulation of chloride ions. This results in endosomal swelling/lysis and release of the genetic materials.[142]

A large number of studies have been devoted to improvement of PAMAM characteristics such as structural flexibility, plasma membrane interactions as decreasing cytotoxicity. In order to alter the structure of the PAMAM dendrimer for gene transfection, regarding the cytotoxicity, the surface of the dendrimer was neutralized by different strategies. As an example, Park et al. synthesized an internally quaternized PAMAM dendrimer with hydroxyl groups on the periphery. The resulting compounds showed reduced cytotoxicity compared to unmodified PAMAM and PEI.[143] As a result of early studies on the role of dendrimer structure in gene delivery, partially degraded dendrimers were investigated. This group of dendrimers showed increased transfection, which was assigned to their increased structural flexibility. This helps DNA to compact and release easier upon pH changes. Currently, a partially degraded PAMAM dendrimer named 'Superfect' is commercially available and often used as a standard for *in vivo* gene transfection studies.[144]

Furthermore, we synthesized fully branched pseudodendrimers (analogue of PPI and PAMAM) using PEI as a core in a two-step procedure. The effect from increasing DB (therefore decreased structural flexibility) and altering PEI molecular weight on the transfection efficiency and cytotoxicity of PEI/PAMAM and PEI/PPI polymers was investigated. The best transfection efficiencies were observed for polymers whose PEI core molecular weight ranged from 6000 to 25,000 $g.mol^{-1}$. The most efficient transfection was demonstrated in NIH/3T3 and COS-7 cell lines, for PEI/PPI with a 60% degree of branching and PEI of MW = 25,000 $g.mol^{-1}$.[145]

After proving the importance of flexibility in the structure for gene transfection by dendrimers, a series of PAMAM dendrimers with triethanolamine (TEA) core were synthesized and evaluated by Wu et al.[146] This structural alteration increases the possibility of accommodating branched structures into the dendrimers. These structurally altered dendrimers have been explored for gene transfection. Such dendrimers form stable nanoscale particles with siRNA that are protected from degradation[147] and better uptaken into the cells.[148] They were capable of transferring the siRNA into the human T and primary PBMC cells, and effectively silencing genes.[149] These dendrimers with the branching groups pointing away from the center were further investigated for *in vitro*/*vivo* gene delivery. In 2011, the

potential of such dendrimers was shown for gene transfection in epithelial and fibroblast cells and more importantly in mouse thymus.[150]

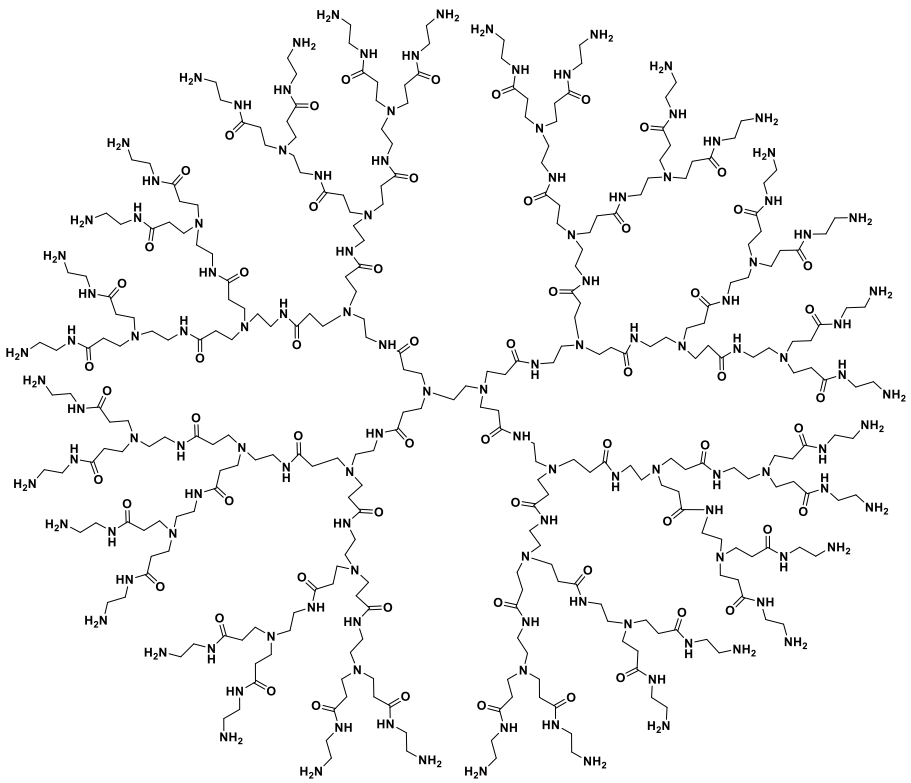


Figure 10. Idealized structure of G4, poly(amidoamine) (PAMAM).

In addition to reducing the toxicity and structural modifications, significant attempts have already been made to increase the plasma membrane interactions with PAMAM dendriplexes. Modification of the dendritic PAMAM with GALA, a membrane-destabilizing peptide, L-arginine, and hydrophobic amino acid such as phenylalanine, or alkyl lipid chains onto the periphery, improved gene transfection in comparison to commercially available PAMAM.[139, 151-152]

After improving the cytotoxicity, structural features, and plasma membrane interactions of PAMAM dendrimers, the next issue to address was the cell specificity. An important strategy for localization of the genetic materials is incorporation of a targeting moiety. A targeted carrier was designed based on generation four [G4] PAMAM dendrimer labeled with quantum dots and decorated with epidermal growth factor (EGF) as the targeting moiety. The resulting nanoparticles were within the cells expressing EGFR more than in the cells lacking the receptors. The targeted vector significantly reduced the expression of yellow fluorescent protein (YFP) as well.[153]

State of the Art, Dendritic Polyglycerolamine

Over the last decade PG-based structures have been tailored for a broad spectrum of applications.[154] In fact, developments like new synthetic approaches to cover a range of dimensions (from nm to several µm), control over the degree of branching, low dispersity, and intrinsic advantages like multiple hydroxyl groups for facile post-modification make PG an ideal platform for a wide spectrum of purposes. The diversity of these applications[155-159] is an indication of PG scaffold's versatility.

Among the vast array of applications, our group recently functionalized dPG with primary amines[160] to achieve efficient complexation and transfection of DNA/siRNA. Therefore, Fischer et al. introduced various synthetic and natural oligoamines to a 10 kDa polyglycerol scaffold via a pH-responsive linker. In this work, a series of core shell architectures were derived from dPG, bearing biodegradable carbamate linkers. These pH responsive core shell architectures were able to silence the expression of different proteins (Lamin, CDC2, MAPK2) using a human epithelial carcinoma cell (HeLaS3) line.[161] Attachment of pH-labile shell to the dPG resulted in a polycationic structures, which were able to form stable complexes with siRNA and release the siRNA through cleavage of the carbamate bond. The degradation of the shell led to a reduction of affinity and consequent release, because the multivalent interactions between the dendritic polyamines and the siRNA were destroyed. *In vivo* degradation of such cationic vehicles is of high significance for efficient therapeutic delivery. In fact, the appropriate degradation of the polymers into low molecular weight breakdown products enables the reduction of cytotoxicity by reducing cumulative cellular exposure time and an easy elimination by excretion pathways.

In the same study, an analog of dPG-NH_2 with high (≥ 90% on a 10 kDa dPG) amine DF was evaluated for siRNA transfection *in vitro* (Figure 11). This nanocarrier showed the best transfection/toxicity ratio. Following the encouraging results of dPG-NH_2 90%, this nanocarrier was further evaluated for siRNA transfection in an *in vivo* set up.[162] In this study, four dPG- or PEI-based nanocarriers, including PEI-gluconolactone (PEI-Glu), (PEI)-polyamidoamine (PEI-PAMAM), polyglyceryl pentaethylenehexamine carbamate (PG-PEHA), and dPG-NH_2 90%, were assessed regarding siRNA complexation, transfection efficiency, and toxicity *in vivo*. Among the synthetic nanocarriers evaluated in this study, the best siRNA transfection efficiency with regard to the toxicity after systemic delivery was observed for dPG-NH_2 90%. Indeed, this work demonstrated that intratumoral and intravenous

administration of dPG-$NH_2$90%/siRNA polyplexes resulted in efficient knock down of the target gene with reduced toxicity compared with other evaluated nanocarriers.[162]

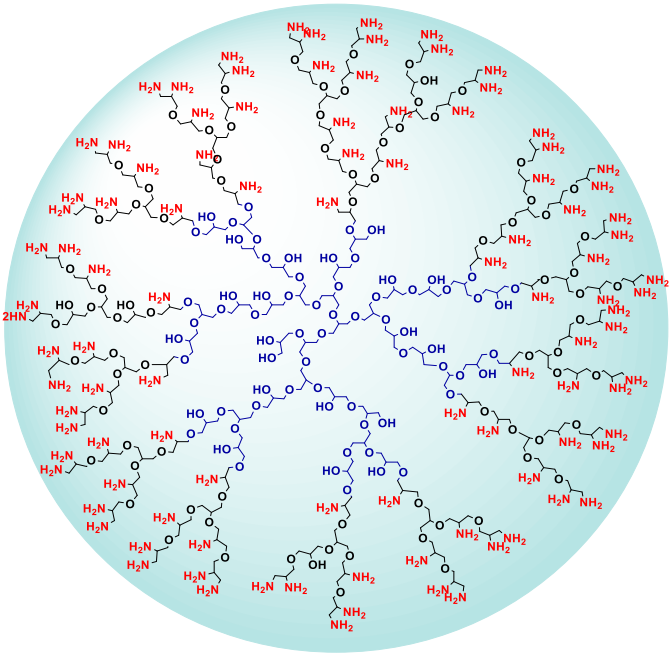


Figure 11. Idealized structure of a 10 kDa dendritic polyglycerolamine (dPG-NH_2) with a high degree of amine functionalization.

Furthermore, Fischer et al. synthesized two PG-based photo-responsive core shell nanocarriers for efficient transfection and controlled release of DNA. Therefore, a 10 kDa PG core was post-modified either with bis-(3-aminopropyl) methylamine (AMPA) or pentaethylenehexamine (PEHA) via a photo-responsive *o*-nitrobenzyl linker. The new photolabile core/shell architecture could efficiently complex dsDNA (21-mer). Furthermore, a fast (within 2 min) triggered release of the nucleic acid cargo from its polyplex upon UV radiation was demonstrated.[163]

1.2.2.2 Lipid-Based Nanocarriers*

1.2.2.2.1 Dendritic Micelles

In most cases, large polycationic dendrimers based on polyamines have been employed as dendritic based carrier systems for nucleic acid delivery. Although such systems can perform highly effective gene delivery, they also exhibit challenging toxicity profiles that can cause problems due to their persistence inside the cells after gene delivery has taken place.[164] Therefore a different approach has been applied using dendron-based architectures in which a

hydrophobic group at the focal point (amphiphiles) encourages self-assembly of the dendrons. This provides a unique way to create supramolecular functional materials "pseudodendrimers."[164] Based on their amphiphilic nature, dendrons of this type are considered as cationic lipids for nucleic acid delivery.[164]

Kataoka et al. reported the synthesis and evaluation of nanosized polymeric micelle structures as promising siRNA carriers. These micelles were prepared to contain three main features: poly(ethylene glycol)-block-poly(L-lysine) (PEGb-PLL) comprising lysine amines as a siRNA binding segment, a hydrophilic nonbinding segment, and cyclo-Arg-Gly-Asp (cRGD) as a cell-surface binding peptide. This formulation resulted in improved control of micelle formation and also increased stability in the blood compartment, while installation of the cRGD peptide improved biological activity and increased gene silencing ability. Addition of the cRGD peptide to the micelle structure resulted in a targeted delivery of siRNA to the site of activity and improved tumor accumulation and cellular uptake following intravenous injection. They could show that stable and targeted micelles inhibited the growth of subcutaneous HeLa tumor models and demonstrate gene silencing in the tumor mass following treatment with antiangiogenic siRNAs.[165]

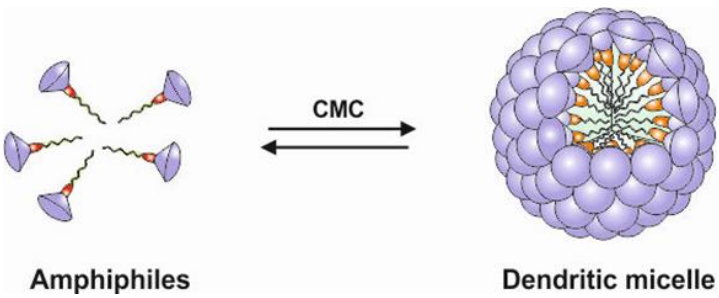


Figure 12. Dendritic micelles prepared by self-assembly of dendritic amphiphiles.

Wagner et al. developed a novel synthetic strategy via click chemistry for site-specific epidermal growth factor (EGF)-PEG functionalization of PAMAM dendrons. In earlier studies, it was reported that EGF ligand modification improved the transfection efficiency of PEI polyplexes 10-100 fold due to the accelerated internalization of polyplexes via epidermal growth factor receptor (EGFR)-EGF interactions.[166] To evaluate the influence of EGF conjugation on the gene transfection efficiency of chain terminal modified PEG-PAMAM dendrons, two polyplex compositions and three different polymer to pDNA weight ratios were tested. Oligoamines with a higher density of secondary amino groups resulted in higher gene transfection efficiency, which was assumed to be due to improved proton sponge capacity. The

PAMAM-pentaethylenehexamine (PAMAM-PEHA) dendron structure was selected as a basis for incorporation of EGF-PEG via click chemistry. The EGF-PEG functionalized (PAMAM-PEHA) dendron polyplexes displayed better gene transfer ability in EGFR overexpressing HuH-7 cells than EGF-free PAMAM polyplexes. Higher generation PAMAM shows high transfection efficiency but causes, at the same time, more cytotoxicity than the low generation ones. To exploit the full potential of PAMAM dendrons as nucleic acid carriers, the impact of chain terminal modification in terms of transfection properties was studied.[166] Lu et al. reported an optimized synthetic procedure of two pH-sensitive amphiphilic carriers, N,N'-bis(oleoylcysteinylhistidyl) lysine ethylenediamine monoamide (EKHCO) and N,N'-bis(oleoylcysteinyl)lysyl]histidylhistidine ethylenediamine monoamide (EHHKCO).[167] These amphiphiles had low critical micelle concentrations and readily formed nanoparticles with pDNA and siRNA. Both carriers showed pH- and concentration-dependent membrane disrupting abilities in free or complexed form with pDNA or siRNA, which resulted in high cellular uptake of pDNA and siRNA and low cytotoxicity at physiological pH. Both EKHCO and EHHKCO resulted in higher gene silencing efficiency in U87 cells than DOTAP.[167]

In comparison to these block-copolymer aggregates, the more defined dendritic amphiphile can play a distinctive role in controlling the biological behavior, e.g., it is possible to control the self-assembly into dendritic micelles through the choice of the hydrophobic group at the dendron focal point.[168] This difference allows micelles to be significantly smaller (5–20 nm) than liposomes and block copolymer micelles and also means that hydrophobic drugs can be encapsulated, leaving the hydrophilic cargo or contrast agents to be appended to the surface.[169] Kataoka et al. have introcduced polymeric micelles based on poly(ethylene glycol)-block-poly(L-lysine) (PEGb-PLL) that in contrast to the aforementioned micelles can incorporate siRNA, as hydrophilic polyanionic molecule, in their cationic core.[165]

One advantage of dendritic micelles over liposomes is their ability to carefully control the carrier size. Recently Peng et al. developed efficient amphiphilic dendrimer vectors for the *in vitro* and *in vivo* delivery of Hsp27 siRNA in a castration-resistant prostate cancer model that showed significant gene silencing and potent anticancer activity.[170] This vector is a lipid/dendrimer hybrid which has a long hydrophobic alkyl chain and a low generation hydrophilic PAMAM dendron with seven tertiary amines in the interior and eight primary amines at the terminals. Its ability to deliver siRNA and induce gene silencing was investigated on the basis of luciferase gene silencing experiments in A549Luc cells. An exceptionally

powerful gene silencing with the specific GL3Luc siRNA was observed, whereas no gene silencing was observed using the nonspecific GL2Luc siRNA bearing 3 mismatches.[170]

Recently our group also presented multivalent oligoglycerol dendrons based on low molecular weight amphiphiles with well-defined structures that express controlled glycine arrays on their surfaces.[164] In this study, the controlled loading of amine content on the surface of the dendritic head-groups using facile chemo-enzymatic and chemical synthetic routes[171] was reported and the structure–activity relationships with respect to the siRNA/DNA complexation, toxicity and transfection profiles with the synthesized polycations were studied. The transfection measurements clearly indicated that the efficiency depended on the number of amine functionalities (glycine loading) on the surface of the dendritic head-group. Enhanced efficiency in terms of siRNA transfection and cytotoxicity was obtained by a second-generation amphiphilic dendrimer with eight amine groups on its surface that has been designated as G2-Octamine. G2-Ocatamine acts as an efficient vector to deliver siRNA inside the cell and achieved potent gene silencing as demonstrated by the knockdown of normalized luciferase activity and also for GAPDH in HeLa cells. The amphiphilic vector is non-toxic even at a higher ratio of N/P 100 and shows little immunogenicity *in vivo*.[164]

1.2.2.2.2 Liposomes

Cationic lipids are amphiphiles that generally consist of three structural domains: the positively charged hydrophilic head group, two lipophilic tails or a steroid moiety, and a connecting linker between the two domains. In aqueous media, self-assembly of dissolved cationic lipid molecules results in liposome formation. Liposomes are sphere-like lipid bilayers with an aqueous core (Figure 13). Today lipid-based materials are one of the most widely used non-viral delivery systems and a number of lipid nanoparticle (LNP) have already entered the clinical trials.[105] Before the application of liposomes for siRNA transfection, these systems were optimized in different studies for DNA delivery purposes.[105, 172] Most of the liposomes that have been used for siRNA delivery have cationic or ionisable lipids. Positively charged lipids acquire several tasks while delivering nucleic acid cargos: encapsulation of negatively charged siRNA, improvement of cellular uptake, and endosomal release.

In 1987, Felgner et al. introduced (N-[1-(2,3-dioleoyloxy)propyl)]-N,N,N-trimethyl ammonium chloride), DOTMA, which has been able to efficiently deliver both DNA and

RNA.[82, 173] Later, in 2006, for the first time, liposomal siRNA formulations were used for RNAi-mediated gene silencing in non-human primates.[174]

In addition to DOTMA, other commonly used cationic lipids are 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), dioctadecyl amido glycin spermine (DOGS), 3,[N-(N1,N-dimethylethylenediamine)-carbamoyl] cholesterol (DC-chol), and 2,3-dioleoyloxy-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethyl-1-propanaminium (DOSPA). Furthermore, Lipofectamine® (Invitrogen, Carlsbad, CA) and HiPerFect® (Qiagen, Hilden, Germany), are well known lipid-based formulations that are used as siRNA delivery benchmarks.

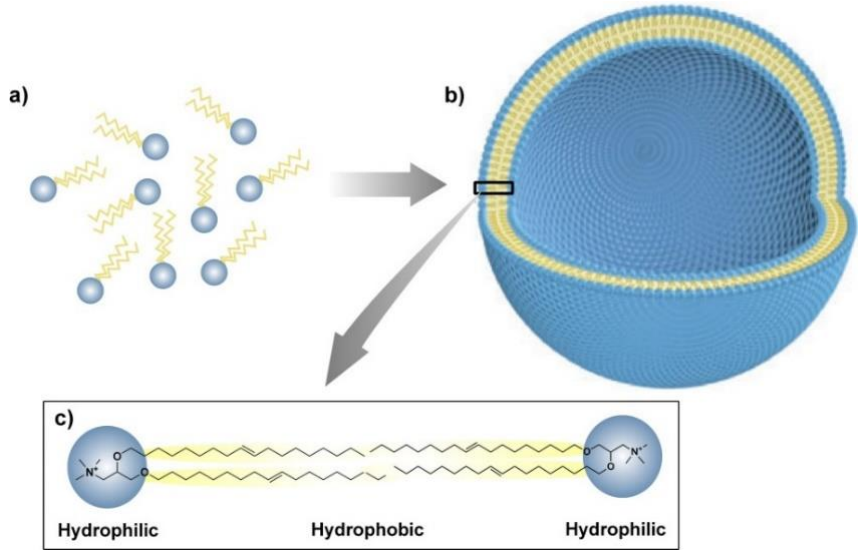


Figure 13. Structural organization of cationic lipids: (a) single cationic lipids, (b) transformation to liposomes, and (c) individual cationic lipid (DOTMA) molecules with their hydrophobic tails facing each other and hydrophilic tails facing towards an external and internal aqueous solution. Figure reprinted with permission from ref. [175].

Since the transfection efficiency can be either affected by structural variations in the hydrophobic domain or the cationic head group, some research groups have investigated such modifications. The hydrophobic tail chains are either linear and saturated or linear and monounsaturated ranging from C_5 to C_{25}, of which oleyl, lauryl, myristyl, palmityl, and stearyl have been the most researched as liposomal vectors.[176] Several studies on DOTMA- or DOTAP based derivatives indicate that gene transfer decreases with increasing the length of hydrophobic chains (C14 > C16 > C18).[177-178] Furthermore, studies from Byk and Sherman et al. found that a decrease in alkyl chain length increased the cytotoxicity in both Hela and NIH 3T3 cell lines.[179] While various cationic head groups have been investigated, nitrogen-based motifs (mainly guanidinium and ammonium groups) are still the most widely used.[180]

Floch et al. attempted to improve transfection efficiency and to reduce toxicity by replacing the ammonium functionality with either phosphonium or arsenium groups. The reduced toxicity was attributed to the increased atomic radii of As and P compared to N, which resulted in the formation of larger cationic complexes with reduced charge densities. Additionally, *in vivo* gene transfer studies for the phosphonium and arsonium derivatives showed up to a 3600-fold increase in gene transfer efficiency compared to DOTAP.[88] The major disadvantage to the *in vivo* use of cationic lipids is their low transfection efficiency, which is attributed to their heterogeneity and instability in physiological, i.e., serum containing environments.[181] Since there are several *in vivo* studies reporting cytotoxicity of cationic lipids upon administration, Lipofectamine and HiPerFect® are therefore virtually the best for *in vitro* experimentation.[181]

1.2.2.2.3 Polymer-Lipid Hybrid Delivery Systems

Amphiphile building blocks align in such a way to liposomes that their hydrophilic heads face the interior and exterior of the supramolecular superstructure, while the interior of the membrane houses the hydrophobic tails.[169] This dual nature enables a liposome to envelope hydrophilic drugs in its interior and hydrophobic drugs in its membrane.[169] Through electrostatic interactions between the positively charged polar head group and a negatively charged phosphate group of the nucleic acid, amphiphilic structures self-assemble with nucleic acids into lipoplexes.[182-183] After the uptake, lipoplexes trigger endosomatic release by provoking membrane perturbation which results in the release of nucleic acids intracellularly.[183-184]

Currently, one of the most successful liposomal delivery techniques involves the use of stable nucleic acid-lipid particles (SNALP), which have been used to therapeutically deliver siRNAs in multiple *in vivo* models.[185] In such particles, the siRNA is surrounded by a lipid bilayer containing a mixture of cationic and fusogenic lipids that have been coated with diffusible polyethylene glycol.[186] In 2005, Breen et al. reported the better *in vivo* knockdown efficacy of siRNA-SNALP complexes than unformulated siRNA, which was targeted to hepatitis B virus (HBV) by intravenous injection into mice carrying replicating HBV. siRNA-SNALP showed reduced plasma clearance and efficient hepatic uptake with dose-dependent, potent (>1 log10) and durable knockdown of circulating HBV DNA levels in a mouse model of infection for up to 6 weeks with weekly dosing.[186] Fedoruk et al. demonstrated hepatic uptake of siRNA-SNALP with specific knockdown of apolipoprotein B (ApoB) for treatment

of hypercholesterolemia in the liver of cynomolgus monkeys.[176] The pharmacodynamic (PD) profile in this study was notable not only for the extent of hepatic ApoB mRNA knockdown (>80%), but also for its translation to systemic lowering of low density lipoprotein cholesterol (LDLc) (by 82%) which persisted for several weeks after a single dose.[176-177] MacLachlan et al. described the development of a potential therapy for Ebola virus (EBOV) infection. The treatment of guinea pigs with a pool of the gene-specific siRNAs delivered by SNALPs completely protected guinea pigs against viral infection and death when delivered following an Ebola virus challenge. Additional experiments demonstrated that seven 2 mg kg^{-1} postexposure doses of nonimmunostimulatory siRNA-SNALP complexes protected all three rhesus macques studied.[178] Anderson at al. have developed lipid-like materials for low-dose *in vivo* gene silencing that are nearly 100 times more efficient at delivering small interfering RNA than previously studied lipid-based carriers.[179] These materials, known as lipidoids, are synthesized through the conjugate addition of alkyl acrylates, alkyl acrylamides, and alkyl epoxides to primary and secondary amine molecules. Lipidoids and lipids share many of the physicochemical properties that cause the formation of liposomes for nucleic acid delivery. However, lipidoids require fewer synthesis steps and are easier to purify, which makes a high throughput combinatorial synthesis of lipidoids and rapid *in vitro* screening of thousands of potential drug delivery candidates possible.[179, 186] Kono et al. developed a novel cationic lipid consisting of a third generation polyamidoamine dendron and two dodecyl chains (DL-G3), which in combination with a fusogenic DOPE achieved efficient transfection of CV1 cells in the presence of serum by synergetic action of the proton sponge effect and membrane fusion.[180] Results showed that the transfection activity of the DL-G3-DOPE lipoplexes was highly dependent on their composition, such as the N/P and DOPE/DL-G3 ratios.[180] Torchilin et al. reported a nanosized micellar siRNA delivery system, into which the GFP-siRNA was incorporated after its preliminary modification with a phosphothioethanol (PE) portion (siRNA-S-S-PE) into 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy-(poly-(ethylene glycol))-2000] (PEG-PE) nanoparticles.[88] The siRNA-phospholipid conjugate can be considered as an amphiphilic copolymer with a hydrophilic siRNA and hydrophobic phospholipid segment with a typical structure of those polymeric amphiphiles which self-assemble into micelles, and in which the siRNA is fully protected against degradation by nucleases for at least 24 h.[88] Furthermore, siRNA could be easily released in the presence of glutathione (GSH) at a concentration mimicking the intracellular levels. In GFP-C166 endothelial cells, mixed GFP-siRNA-S-S-PE/PEG-PE micelles down-regulated the GFP production 50-fold more effectively than free siRNA. Furthermore, those micelles did not show

any of the cytotoxic side effects typical for siRNA delivery systems that are based on electrostatic association of siRNA with cationic carriers.[88]

1.3 Targeted Delivery Approaches

1.3.1 The "Magic Bullet"

At the beginning of 20th century, "Paul Ehrlich," the founder of chemotherapy, introduced the concept of drug targeting in an article with the following title: "Aus Theorie und Praxis der Chemotherapie." This concept is better known as the "Magic Bullet." In his point of view, the "magic bullet" has two main components: One component recognizes and binds to the target and the second one is responsible for the therapeutic effect.[187] Although pharmaceutical agents can be coupled directly to the targeting agents like immunotoxins[188] to achieve targeting, new approaches based on Ringsdorf's vision of polymeric drug are possibly a better alternative. In 1975, Helmut Ringsdorf proposed a rational model for polymeric drugs based on covalent linkage of therapeutic low molecular weight drug, solubilizing agent, and targeting moiety to a polymeric backbone (Figure 14).[189] Targeting agent assists in biorecognition, whereas introducing a cleavable linker between the therapeutic drug and polymeric backbone ensures the release of the drug after cellular uptake in the site of function.

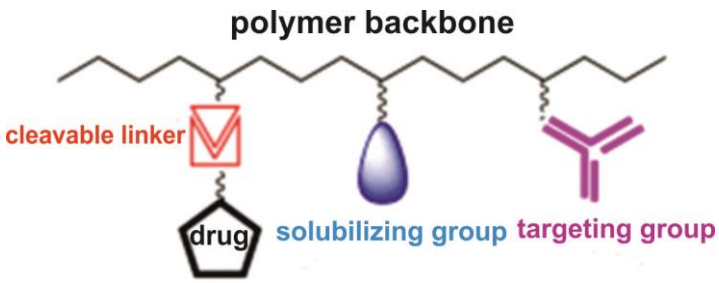


Figure 14. Schematic representation of a polymeric drug according to Ringsdorf's concept (1975). Figure was reprinted with permission from ref. [159]. Copyright (2006). Wiley-VCH Verlag, Weinheim.

In general, successful targeting of pharmaceuticals and their nanocarriers can be achieved by different approaches. These approaches do not necessarily involve targeting moieties.[187] For example, specific physiological features of the target site like tumor microenvironment can be used to achieve targeting.[187] Furthermore, size and surface characteristics of nanocarriers can be tuned in a way to avoid their uptake by reticuloendothelial system (RES), thereby

improving their direction to the site of function.[190] Based on EPR, a number of nanocarriers like polymer-protein conjugates (Styrene maleic anhydride-neocarzinostatin (SMANCS) or PEG-L-asparaginase) or liposomes have been developed and reached the market in the 1990s.[191] Nonetheless, passive tumor targeting is influenced by a number of factors including size and type of the tumors. Furthermore, EPR is intrinsically a random approach[191] and is less effective in large tumors due to the avascular regions or in small metastases at their pre-angiogenic stage.[192] Longer circulation time and EPR effect will only increase the local concentration of the nanocarriers in the vicinity of the cells. To further improve the therapeutic effect of the drugs including nucleic acid and antineoplastic drugs, internalization into the interior of the target cells by active targeting strategies is essential. These delivery approaches are based on utilizing nanocarriers with targeting agents that their receptors or antigens are expressed uniquely or overexpressed on tumor cells.[191]

1.3.2 Targeting Agents

1.3.2.1 Non-antibody Targeting Agents

One classification of targeting agents is into three main groups (Figure 15): receptor ligands (peptides, vitamins, and carbohydrates), nucleic acids (aptamers), and proteins (mainly antibodies and their fragments). Different receptors have been employed for drug targeting approaches, which include vascular receptors like integrins[193] and vascular endothelial growth factor receptor,[194] plasma protein receptors like low-density lipoprotein receptor[195] and transferrin receptor,[196] peptide receptors like Luteinizing-hormone-releasing-hormone receptor,[197] receptors for growth factors and vitamins like folate receptors,[198-199] epidermal growth factor receptors,[200] and carbohydrate receptors like galectins,[201] selectin,[202] and hyaluronic acid receptors.[192]

Aptamers are short single-stranded DNA or RNA nucleotides that have found applications as targeting molecules.[203] Aptamers may be utilized to target a wide range of large molecules including transmembrane, intracellular and soluble proteins or carbohydrates, and small molecules.[191, 204]

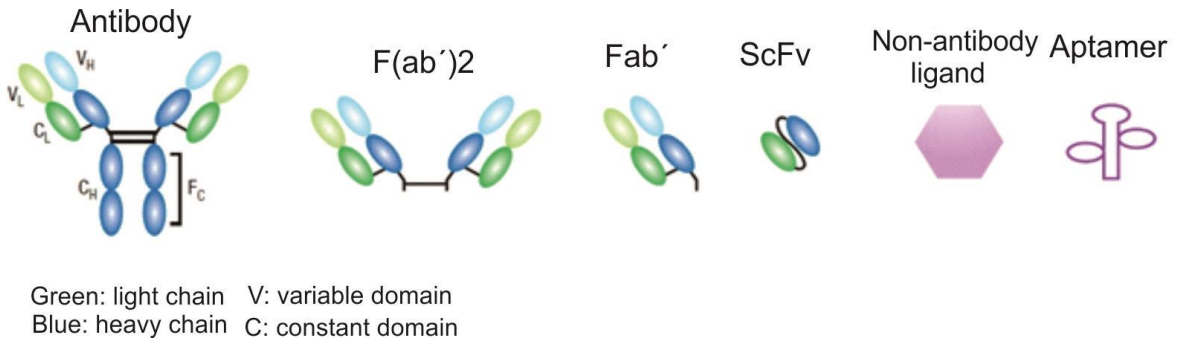


Figure 15. Representation of a monoclonal antibody (mAb) and its fragments, receptor ligand, and an aptamer as targeting agent. Figure was reprinted with permission from ref. [191]. Copyright (2007) Nature Publishing Group.

1.3.2.1.1 Targeting $\alpha_v\beta_3$ Integrins with RGD

Small tumors at a size of about 2 mm^3 start to induce the formation of new blood vessels from the already existing vasculature in a complex process. This process is called angiogenesis.[205] Several groups of adhesion molecules including members of integrin, cadherin and selectin families are involved in angiogenesis.[206] Integrins are extracellular cell adhesion receptors that are involved in several processes.[207-208] Particularly, integrins are involved in adhesive events that happen during cancer stages like growth, progress, transformation, and metastasis.[208] Although various types of integrins are involved in angiogenesis, $\alpha_v\beta_3$ play a significant role in angiogenesis regulation.[209] $\alpha_v\beta_3$ receptors are over expressed on tumor endothelium, tumor blood vessels and cancer cells in many cell lines[210] and therefore are an attractive target for cancer therapy and diagnosis.

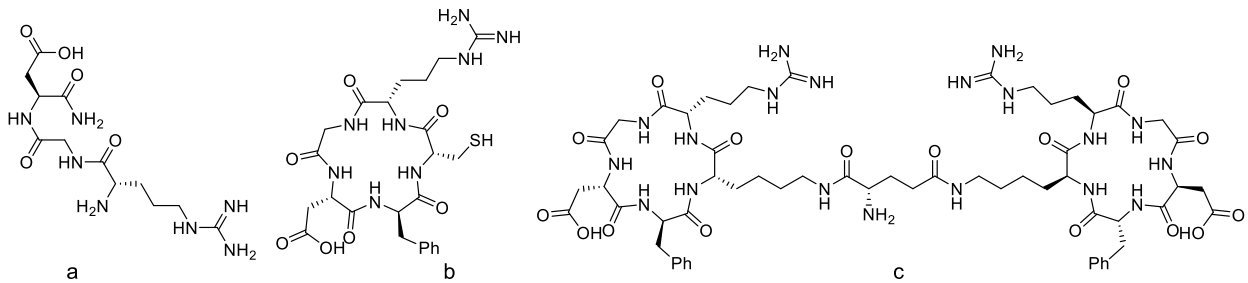


Figure 16. a) Arginine-glycine-aspartic acid (RGD) sequence, b) a cyclic monovalent RGD (RGDFC), and c) a cyclic bivalent RGD peptide, E-[c(RGDfK)$_2$].

Arginine-glycine-aspartic acid (RGD) sequence, is known for its preferentially binding to $\alpha_v\beta_3$ integrins. The sequence of RGD was first found in fibronectines[211] and later discovered as a peptide sequence in various natural $\alpha_v\beta_3$ integrin binding ligands. RGD as a cell binding motif, has been employed for selective imaging of solid tumors especially at the early

stages.[212] Although RGD containing peptides can be found as linear or cyclic structures, cyclic derivatives of RGD have the advantages of proteolysis resistance (due to their less structural flexibility) and higher binding affinity to the integrin receptors.[213] Monovalent, bivalent, and multivalent pentapeptide derivatives of cyclic RGD (Figure 16) have been conjugated to near infrared (NIR) dyes or radiotracers for diagnostic purposes.[214]

One targeting strategy using RGD is to introduce the peptide or its peptidomimetics to the surface of nanocarriers for therapeutic and diagnostic purposes.[215] In order to bring nucleic acids in enough quantities and for a certain time in the target tissue, ligands such as RGD have been grafted on the surface of nucleic acid delivery systems. Nanocarriers including liposomes,[216] micelles,[217] and nanoparticles[218] have been decorated with RGD to take advantages of both passive and active targeting. Various polycationic nanocarriers including PEI,[219] chitosan,[220] and poly-L-lysine[221] have shown enhanced transfection results after grafting RGD on their surface. Similar promising results have also been demonstrated using RGD-decorated dendrimers.[222-223] Furthermore, these studies revealed evidence of receptor mediated endocytosis and direct interaction of RGD-functionalized nanocarriers with tumor tissues, in addition to enhancement of gene/siRNA transfection.

1.3.2.2 Antibodies as Targeting Agent

Immunoglobulin (Ig) antibodies are heterodimeric glycoproteins composed of two heavy (H) and two light (L) chains. Igs according to the isotype of their heavy chains are divided (in mammals) into five subclasses: IgM, IgA, IgD, IgG, and IgE.[224] Light and heavy chains in IgGs have two different domains according to their function: variable (V) and constant (C) domains (Figure 15). Variable domains recognize antigens, whereas the constant domains are responsible for functions like Fc receptor binding. Enzyme such as Papain and Pepsin can split IgG into various fragments.[225] Papain splits IgG into two Fab fragments and a single Fc fragment while Pepsin digest IgGs into a single dimeric $F(ab)_2$ and a Fc fragment (Figure 15, antibody fragments). One more fragment of Igs is a single chain Fv fragment (scFvs) that is constructed by linking the variable regions of heavy and light chains via a short peptide linker. Antibody fragments show similar binding characteristics as they parent antibodies.[224] Monoclonal antibodies (mAbs) may be utilized for targeting approaches either as a whole or antibody fragments.

Milstein and Kohler introduced mAb for cancer targeting in 1981.[226] Antibodies can target cancer and other diseases with various mechanisms including evoking immune responses

through complement-mediated cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) or directly inducing apoptosis.[227] Due to the advances in antibody engineering in the 1990s and early 2000s, several mAbs including rituximab (Rituxan)[228], trastuzumab (Herceptin)[229], cetuximab (Erbitux®) bevacizumab (Avastin)[230] emerged for targeting different cancer types. In addition to mAbs, antibody drug conjugates (ADC) have found diverse therapeutic applications.[231] ADCs exploit the antigen-selectivity of antibodies to achieve selective delivery into tumor cells. Nonetheless, the early studies on the development of ADC did not show any significant therapeutic benefits.[232] Application of murine or chimeric antibodies that evoke immunogenic responses and the lack of appropriate target antigen selection have been considered the main reasons of the failure.[233-234] Today, two drugs based on ADC including ado-trastuzumab emtansine (Kadcyla®) and brentuximab vedotin (Adcetris®), have been marketed in the United States and many more have reached clinical studies.[232] Additionally, site-specific conjugation of radionuclide to mAb generates Radionuclide Antibody Conjugates (RAC) for therapeutic and diagnostic purposes. Currently two RAC, ibritumomab tiuxetan (Zevalin®)[235]and tositumomab (Bexxar®), that target anti-CD20 mAbs have been validated for clinical treatment of lymphoma.[236] mAb can be prepared to target antigens with high selectivity. Nevertheless, antibodies are generally expensive, instable, time consuming to produce, and inconvenient to store and handle.[235] Non-antibody ligands, in contrast, have the advantage of being available, easy to handle and manufacture, although they possess much less selectivity.

1.3.2.2.1 Bispecific Antibodies for Targeting Purposes

Bispecific antibodies (bsAb) have the specificity of two antibodies within one single molecule and therefore can simultaneously address two distinct antigens or epitopes.[113] bsAbs can be prepared either by chemical conjugation of two purified mAb or fusing two hybridomas.[237]

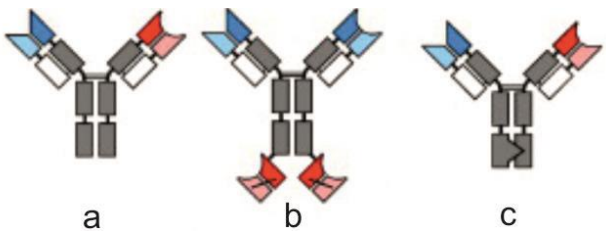


Figure 17. Schematic representation of two bivalent (symmetric (a) and asymmetric (c)) and one tetravalent (b) bsAb. Figure was adapted with permission from ref. [237]. Copyright (2015) Elsevier.

Coupling of antigen binding sites of two different antibodies with diverse strategies can generate bivalent (symmetric or asymmetric) and tetravalent bsAbs or other molecules like non-immunoglobulin fusion proteins (Figure 17).[237] Owing to genetic engineering, a wide range of recombinant bsAb formats is currently available for different applications, and more than 20 recombinant bsAbs have reached clinical trials.[237] Because of their structural feature, bsAbs may be used to selectively deliver cells, such as immune effector cells[238] or therapeutic agents, to the tumor site. In general, therapeutic bsAbs have three different action modes (Figure 18a-c): crosslinking of T cells or natural killer (NK) cells to tumor cells, receptor signaling interference, and antibody-mediated forced assembly of coagulation entities.[237] A number of tumor-associated antigens like clusters of differentiation 19 (CD19), CD20, human epidermal growth factor receptor 2 (HER2), and epithelial cell adhesion molecules (EpCAM) have been recruited for redirecting immune effector cells to tumors.[239] These antigens exist on various immune effector cells including T-cells, natural killer (NK) cells, monocytes and neutrophils.

The initial clinical evaluations of bsAbs were rather disappointing due to the low efficiency and safety issues.[240] However, the European Medicines Agency (EMA) approved in 2009 the first bsAb, catumaxomab (Removab®) (Fresenius Biotech GmbH/ Trion Pharma) for treatment of patients with malignant ascites.[241]

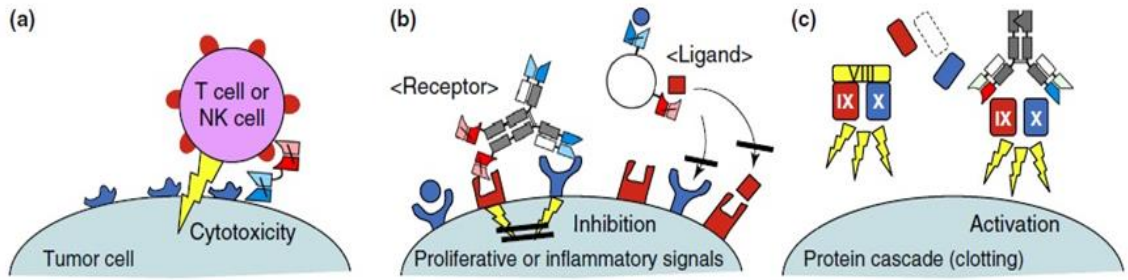


Figure 18. Therapeutic bispecific antibodies have three different modes of action. a) Crosslinking of T cells or natural killer (NK) cells to tumor cells, b) Receptor signaling interference, c) Antibody-mediated forced assembly of coagulation entities Figure was adapted with permission from ref. [237]. Copyright (2015) Elsevier.

Recently, in a different approach, bsAbs have been used in targeted payload delivery. This strategy is based on simultaneous recognition of the cell surface antigens and a hapten by bsAb.[242-243] bsAbs that engage in the binding with a cell-surface marker and haptens have been already utilized to deliver imaging agents by pretargeting strategies.[244-246] In the first

strategy, payloads are conjugated to a hapten that can form a complex with one recognition site of the bsAb. This way, targeted delivery is achieved by binding the hapten-binding bsAb to the target antigens on tumor cell. In this strategy, chemical modification of the antibodies as a targeting agent can be avoided. Chemical modification may cause inactivation of the antibodies as a targeting agent and/or generation of immunogenic sites within the antibody.[243]

1.3.2.2.2 SNAp-tag Technology for Targeting Purposes

An important approach to study the function of proteins and antibodies in living cells is "protein labeling" by chemical techniques.[247] Conventional strategies to label proteins in living cells rely on random modification of functional groups such as reducing the sulfhydryl groups of cysteine residues or using amines on the lysine side chains. However, this method results in heterogeneous products with no control over conjugation sites and ratio and therefore different therapeutic efficacy and safety profiles.[248] Furthermore, proteins that are randomly modified may show unpredictable stability, solubility and binding affinity.[249] Additionally, site specific labeling of proteins by adding or removing particular reactive groups using genetic engineering needs careful and laborious screening of the suitable target positions.[250-251] Thus, one alternative promising strategy is based on the expression of the desired protein as a fusion protein with an additional short polypeptide, the so-called tag, which serves as a label acceptor group.[252-253] A number of factors influence the feasibility and applicability of this approach, including the size of the tag, the speed of the labeling, specificity, and availability for a broad range of probes.[254] Recently, Keppler and colleagues introduced an attractive method for labeling proteins and recombinant antibody fragments[249] by fusing them to an engineered version of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), briefly called SNAP-Tag.[254]

While labeling, AGT recognizes O^6-alkylated guanine residue on DNA or other substrates containing O^6-benzylguanine (BG) and then irreversibly transfers the substituted benzyl group to its reactive cysteine residue and releases free guanine and forms a stable thioether bond (Figure 19). Site-specific conjugation of the effector molecules to proteins with SNAP-Tag technology has several advantages. Conjugation is performed under simple reaction conditions, is fast (1-2 h), and works with defined (1:1) stoichiometry (1 tag reacts with 1 BG-modified substrate). Moreover, a number of BG-functionalized molecules are available for coupling to desired protein.[255-256] Indeed, SNAP-tag technology is a feasible platform for functionalizing

the desired proteins with a wide range of compounds like dyes, biotin, and other effector molecules (Figure 19). Promising applications of this technology range from in-cell labeling of tagged proteins[254] to immobilization of proteins on chip surfaces.[257]

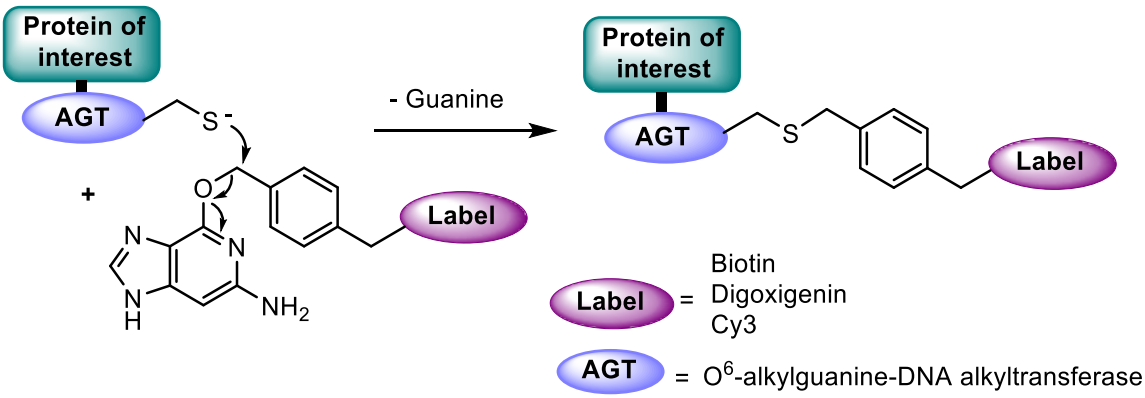


Figure 19. General mechanism of AGT fusion proteins labling by BG-functionalized molecules. Figure reproduced with permission from ref. [254]. Copyright (2004) Elsevier.

Recently, SNAP-Tag technology was used to specifically deliver a multifunctional prodrug immuneconjugate based on dPG to a cancer cell lines expressing epidermal growth factor receptor (EGFR).[157] In this work, SNAP tag was used to conjugate a scFv antibody fragment to a dPG prodrug nanocarier and yielded a selective delivery of immunoconjugates and targeted toxicity only on the cells over expressing EGFR.[157] Recombinant single-chain antibody fragment (scFv), which was used for targeting in this study, has multiple advantages including small size, high specificity, and lack of immune effector function.

*The highlighted sections have been published as: F. Sheikhi Mehrabadi, W. Fischer, R. Haag, *Current Opinion in Solid State and Materials Science* **2013**, *16*, 310-322.

2 Objectives

Short interfering RNA (siRNA) has the potential to silence virtually any gene in the cytoplasm and therefore addresses many diseases on the molecular level. Nonetheless, unmodified siRNA, is not efficiently uptaken by the cells and rapidly degraded upon uptake in the host cells. Therefore, the therapeutic potential of siRNA, is considered to be "far reaching."[105] siRNA delivery issues like poor cellular uptake and enzymatic degradation can be partially addressed by diligent modification of siRNA sequence and/or structure or direct delivery approaches. Nevertheless, *in vivo* delivery is still an unsolved problem to bring the full potential of siRNA to the clinic and to access specific tissues. Materials that are used for siRNA delivery should fulfill multiple criteria: In general, they should be able to (i) protect siRNA against enzymatic degradation, (ii) improve its cellular uptake and (iii) tissue specificity, (iv) increase the siRNA half-life in the blood stream, (v) ideally have no toxicity, (vi) be able to escape from the endosome, and (vii) release siRNA into the cytosol.

Over the last few decades, many polymeric and lipid-based delivery systems have been developed to overcome challenges, such as unfavorable pharmacokinetics and the lack of tissue selectivity associated with low molecular weight and macromolecular drugs like siRNA. Among polymeric carriers, dendritic structures including dendrons, dendrimers, and hyperbranched polymers have shown promise for delivery purposes. As the versatility and biocompatibility of dendritic polyglycerol (dPG), particularly for biomedical applications, have been proven, the major goal of this work will be to use dPG scaffold and alter it in such a way to achieve efficient delivery of drugs, especially siRNA *in vitro* and *in vivo*.

A dendritic polyamine based on dPG, with 90% amine degree of functionalization (DF), has shown successful knockdown of several proteins *in vitro*[258] and efficient siRNA transfection *in vivo*.[162] In spite of high transfection efficiency, the toxicity of dPG-NH_2 90% at relevant therapeutic concentrations heightens and limits its further therapeutic application. Therefore finding a compromise between efficiency and cytotoxicity of dPG-NH_2 is of significant interest. In general, optimization of cationic polymeric carriers to acquire a balance between cytotoxicity and efficiency is a key challenge. Therefore, a major part of this work will focus on the structure-activity function of dPG-NH_2 analogues with different dPG core molecular weights and DF of amine.

In the first part of this work, several 10 kDa dPG-NH_2 analogues with increasing DF of amine will be synthesized and the effect from altering dPG-NH_2 DF on nucleic acid

complexation will be studied. Furthermore, the resulting polyplexes of dPG-NH_2 derivatives/siRNA will be assessed regarding cytotoxicity and knockdown efficiency *in vitro* and *in vivo*. In a combinatorial approach, we shall employ, parallel to the experimental approach, theoretical studies to gain a deeper understanding of the interactions between dPG-NH_2 analogues and a 21-base pair DNA model.

Within the second part of this work, dPG-NH_2 molecules with a DF of 50, 70, and 90% will be synthesized and compared with their analogues of higher (43 kDa) dPG molecular weight and the same DF. The comparison including complexation capability, cytotoxicity and efficiency of transfection *in vitro* and *in vivo*. Furthermore, the corresponding polyplexes of all dPG-NH_2 analogues with siRNA will be carefully analyzed regarding physicochemical characteristics like size and surface charge.

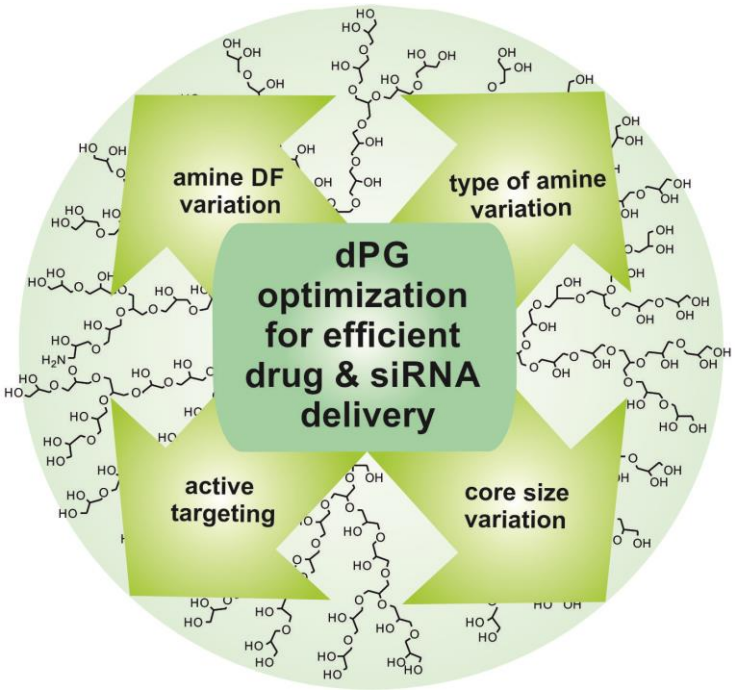


Figure 20. Illustration of dPG scaffold's optimization for efficient drug and siRNA delivery.

The next part of this work will follow the improvement of the toxicity profile of dPG-NH_2 while maintaining its transfection effectiveness. Thus, two relevant basic amino acids, namely arginine and histidine, will be introduced to the primary amines on dPG scaffold. Both arginine and histidine amino acids are known to enhance transfection efficiency of other cationic carriers.[259-260] In the first step, synthesis of amino acid functionalized dPG (AAdPG) will be carried out following a physicochemical characterization and biological evaluation of their corresponding polyplexes with siRNA. Overall, in this part of the work, the effect from

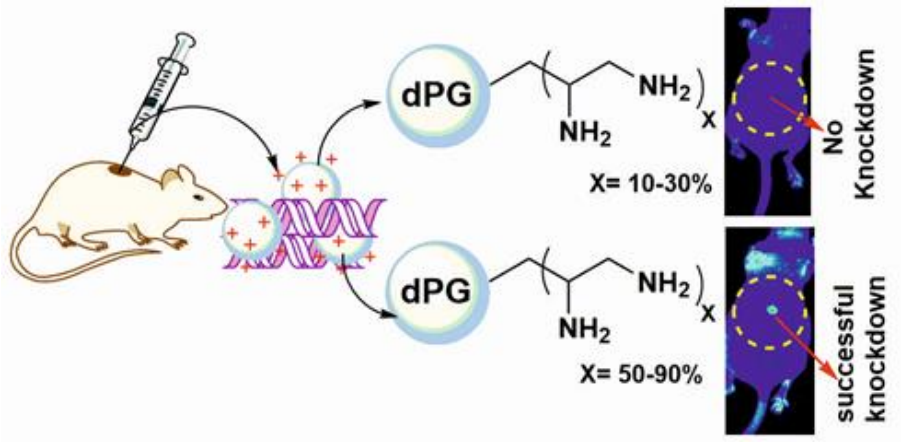
introducing two amino acids with a biocompatible nature to enhance the safety of final carriers will be explored and the results will be compared with bare dPG-NH_2.

As outlined in the introduction, active targeting approaches can bring enough quantities of therapeutic drugs to the site of action to enhance their therapeutic effects. In the last part of this work, a novel targeting strategy based on bispecific antibodies (bsAb) will be utilized for targeted delivery of dPG-based prodrug conjugates. This section will investigate the feasibility and applicability of bsAbs for selective delivery of anticancer drugs into tumor tissue. Therefore, the synthesis, characterization, and *in vitro* biological evaluation of dPG-based prodrug conjugates for tumor selective drug delivery will be followed.

3 Publications and Manuscripts

In this chapter the published articles are listed and the contributions of the author are described.

3.1 Structure-Activity Relationship Study of Dendritic Polyglycerolamines for Efficient siRNA Transfection



This work was published as follows:

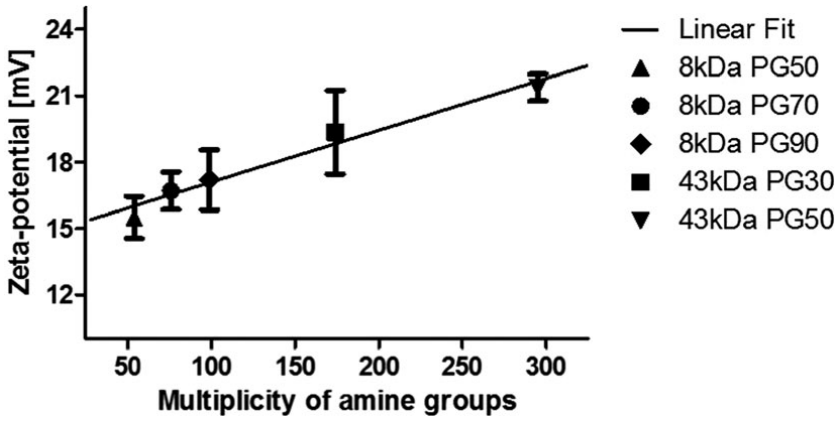
Fatemeh Sheikhi Mehrabadi, Ole Hirsch, Reiner Zeisig, Paola Posocco, Erik Laurini, Sabrina Pricl, Rainer Haag, Wolfgang Kemmner and Marcelo Calderón, *RSC Adv.* **2015**, *5*, 78760-78770.

http://dx.doi.org/10.1039/C5RA10944B

Author's contributions

- Synthesis of dendritic polyglycerolamine analogues
- Full Characterization of dendritic polyglycerolamine analogues
- Preparation of the manuscript

3.2 Optimized effective charge density using polyglycerol amines leads to strong and target specific knockdown efficacy



This work was published as follows:

Anna Maria Staedtler, Markus Hellmund, **Fatemeh Sheikhi Mehrabadi**, Bala N. S. Thota,

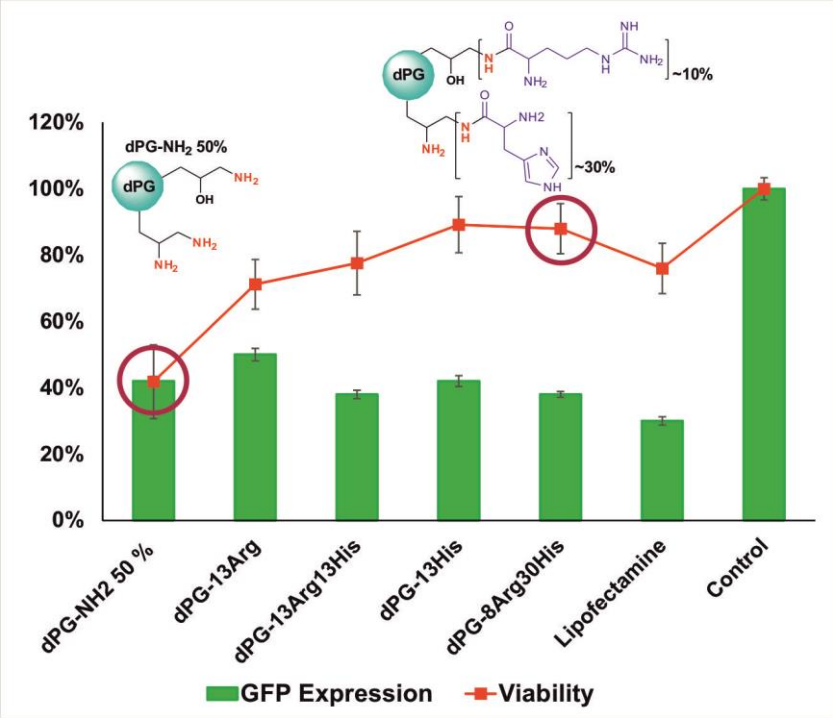
Thomas M. Zollner, Markus Koch, Rainer Haag, and Nicole Schmidt, *J. Mater. Chem. B*, **2015**, *3*, 8993-9000.

http://dx.doi.org/10.1039/C5TB01466B

Author's contributions

- Synthesis of dendritic polyglycerolamine (low molecular weight analogues)
- Full characterization of dendritic polyglycerolamine analogs and their resulting polyplexes

3.3 Multivalent dendritic polyglycerolamine with arginine and histidine end groups for efficient siRNA transfection



This work was published as follows:

F. Sheikhi Mehrabadi, H. Zeng, M. Johnson, C. Schlesener, Z. Guan, and R. Haag, *Beilstein J. Org. Chem.*, **2015**, *11*, 763–772.

http://dx.doi.org/10.3762/bjoc.11.86

Author's contributions

- Synthesis of amino acid functionalized dPG analogues
- Full characterization of amino acid functionalized dPG analogues and their corresponding Polyplexes
- Analysis of all data and preparation of the of the manuscript

BEILSTEIN JOURNAL OF ORGANIC CHEMISTRY

Multivalent dendritic polyglycerolamine with arginine and histidine end groups for efficient siRNA transfection

Fatemeh Sheikhi Mehrabadi[1], Hanxiang Zeng[2], Mark Johnson[2], Cathleen Schlesener[1], Zhibin Guan[*2] and Rainer Haag[*1]

Full Research Paper

Open Access

Address:
[1]Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany and [2]Department of Chemistry, University of California, 1102 Natural Sciences 2, Irvine, California 92697-2025, USA

Email:
Zhibin Guan[*] - zguan@uci.edu; Rainer Haag[*] - haag@chemie.fu-berlin.de

* Corresponding author

Keywords:
arginine; dendritic polyglycerolamine; histidine; multivalent vector; siRNA delivery

Beilstein J. Org. Chem. **2015,** *11,* 763–772.
doi:10.3762/bjoc.11.86

Received: 24 February 2015
Accepted: 28 April 2015
Published: 13 May 2015

This article is part of the Thematic Series "Multivalency as a chemical organization and action principle".

Associate Editor: H. Ritter

Abstract

The success of siRNA-based therapeutics highly depends on a safe and efficient delivery of siRNA into the cytosol. In this study, we post-modified the primary amines on dendritic polyglycerolamine (dPG-NH_2) with different ratios of two relevant amino acids, namely, arginine (Arg) and histidine (His). To investigate the effects from introducing Arg and His to dPG, the resulting polyplexes of amino acid functionalized dPG-NH_2s (AAdPGs)/siRNA were evaluated regarding cytotoxicity, transfection efficiency, and cellular uptake. Among AAdPGs, an optimal vector with (1:3) Arg to His ratio, showed efficient siRNA transfection with minimal cytotoxicity (cell viability ≥ 90%) in NIH 3T3 cells line. We also demonstrated that the cytotoxicity of dPG-NH_2 decreased as a result of amino acid functionalization. While the incorporation of both cationic (Arg) and pH-responsive residues (His) are important for safe and efficient siRNA transfection, this study indicates that AAdPGs containing higher degrees of His display lower cytotoxicity and more efficient endosomal escape.

Introduction

Since the discovery of RNA interference (RNAi) and awareness of its role in posttranscriptional gene silencing, tremendous efforts and capital have been devoted to the development of therapeutics based on this pathway [1]. So far, there are at least 22 RNAi-based drugs in clinical trials and many more are being developed [1]. Although a direct delivery of "naked" siRNA or chemically modified oligonucleotides [2] has been studied, delivery vectors are typically required for efficient siRNA delivery in vivo due to unmodified siRNA's low stability towards endogenous enzymes, poor cellular uptake, and its immunogenic potential [3].

Among the different polymeric vectors, polycationic dendrimers and related structures have found wide application

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

in gene/siRNA delivery [4]. This is because the synthesis of dendrimers and dendritic polymers under controlled conditions results in defined structures with low dispersity. Moreover, the tree-like structure of such polymers provides multivalent positions for functionalization and interaction with DNA/siRNA.

Dendritic polyglycerol (dPG) can be synthesized on a kilogram scale by a one-step, ring-opening polymerization of glycidol with controllable sizes and degrees of branching [5]. Additionally, dPG has multiple groups for further functionalization, high chemical stability, and good biocompatibility in vitro and in vivo [6-8]. All these characteristics make dPG an ideal scaffold for a broad range of applications from anti fouling [9] to biomedical purposes [6] such as anti-inflammatory [10] and anticancer therapy [11,12].

Previously a number of cationic polymers like chitosan [13-15], PEI [16], and PAMAM [17] have been post-modified with histidine (His) or arginine (Arg) groups. The introduction of histidine groups has been beneficial for improving the endosomal release properties [18], and conjugation of arginine groups has enhanced the transfection efficiency of cationic carriers [19,20]. Since the incorporation of either amino acid alone can improve siRNA transfection, we hypothesized that functionalization with both Arg and His may have a synergistic effect on siRNA transfection. Moreover, the biocompatible nature of the amino acids can possibly decrease the cytotoxicity of the resulting vectors. Furthermore, Arg and His groups interact in histones, as natural DNA binding proteins, via their positive residues with the negative phosphates groups of the DNA [21]. Here, we chose dendritic polyglycerolamine (dPG-NH_2) with moderate amine loading (50% of all hydroxy groups on a 10 kDa dPG core) and introduced both amino acids via amide coupling to mimic DNA histones interactions.

In a recent study, our group demonstrated the potential of dPG-NH_2 with high amine loading (≥90%) for siRNA delivery in vivo [22]. Moreover, it has been shown that dPG-NH_2 90% is able to efficiently downregulate the formation of several proteins in vitro [23]. In spite of its high efficiency, the therapeutic window of dPG-NH_2 90% is small and the cytotoxicity increases at higher concentrations which limits its further application. Here, we compare the potential of multivalent amino acid functionalized dPGs (AAdPGs), for siRNA transfection with dPG-NH_2 90%. The initial in vitro results indicated that AAdPGs were capable of mediating efficient siRNA delivery to NIH 3T3 cells and induced comparable gene silencing to both dPG-NH_2 90% and lipofectamine RNAiMAX. In comparison with dPG-NH_2 90%, the new vectors showed reduced cytotoxicity and enhanced siRNA binding.

Results and Discussion

Functionalization of dPG-NH_2 with arginine and histidine

Amino acids have been implemented for the improvement of gene/siRNA transfection using various strategies. Beside peptide dendrimers [24,25], another strategy is to functionalize the periphery groups on cationic vectors such as PLL [26], PEI [16], and PAMAM [19]. In the current study, ≈50% of all hydroxy groups on dPG (M_n = 8.4 kDa, PDI = 1.7) were converted to amino groups according to an earlier published procedure (Scheme S1, Supporting Information File 1) [27]. The high density of amines on dPG facilitates the introduction of groups like amino acids by feasible strategies like amide coupling. Here, we coupled both Arg and His groups in different ratios to dPG-NH_2 via the latter strategy (Scheme 1). By introducing Arg on the dendritic scaffold, this group can serve as a complexing agent and the surplus guanidium groups with high affinity to phosphate groups can interact with the cell membrane and improve the cellular uptake [28]. Additionally, the histidine groups can facilitate tackling the endosomal release problem by improving the polyplexes's buffering capacity [18]. Moreover, arginine and histidine groups can form intermolecular hydrogen bonds with cell surface phosphate groups. These interactions can induce cellular uptake of AAdPG polyplexes. Therefore, four cationic vectors were prepared by Arg and His functionalization of the dPG scaffold. The list of all synthesized samples is presented in Table 1. The samples were named based on their degree of Arg and/or His functionalization on the polymeric backbone (dPG). The functionalization degree for each polymer was determined by comparing the peak integral of either the methylene groups of arginine in high field or the imidazole ring of histidine in the aromatic area (7.2–8.7 ppm) with the assignable dPG backbone signal (Supporting Information File 1).

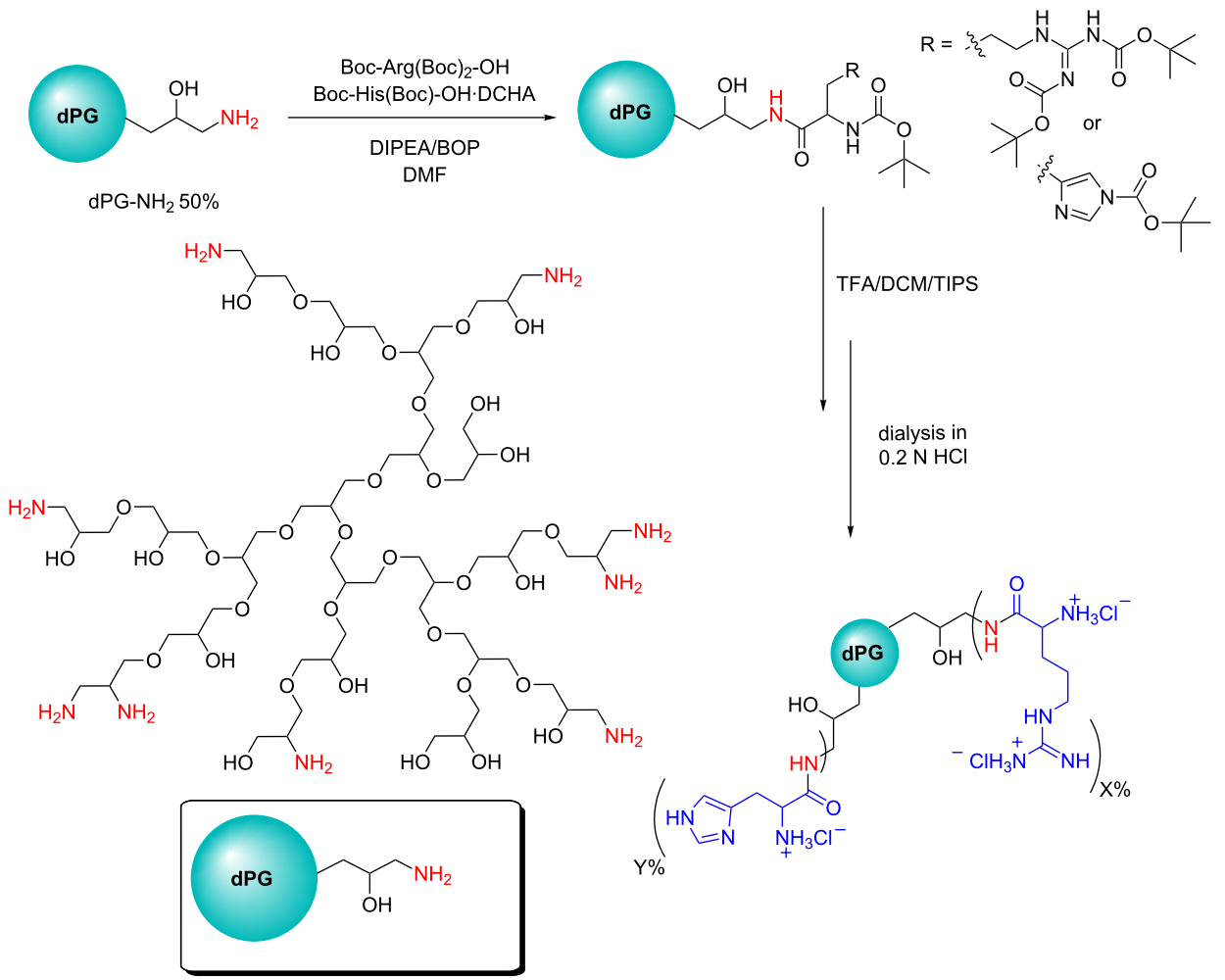
Variable composition of arginine and histidine on dPG-NH_2 50%

To investigate the effect from introducing both His and Arg to dPG backbone on transfection efficiency, cytotoxicity, and cellular uptake, two vectors were synthesized with equal (dPG-13Arg13His) and different (dPG-8Arg30His) composition ratios of both amino acids. Moreover, two further vectors with either Arg (dPG-13Arg) or His (dPG-13His) were prepared to examine the effect of each amino acid alone. The summary of all dPG-based vectors is shown in Table 1.

siRNA Binding

The ability of AAdPGs to form complexes with siRNA was examined by agarose gel electrophoresis retardation assay. The electrophoretic mobility of the siRNA should have been reduced or completely eliminated as a result of complexation

Beilstein J. Org. Chem. **2015,** *11,* 763–772.



Scheme 1: Synthesis of multivalent arginine and histidine functionalized dPG-NH_2 50%. The depicted dPG-NH_2 represents only a small idealized fragment of a 10 kDa molecule.

Table 1: Summary of AAdPG vectors and their corresponding polyplex characterization.

Compound	Zeta potential (mV)[a]	diameter (nm)[b]	PDI[c]	(Arg) %[d]	(His) %[d]	Arg:His
dPG-NH_2 50%	10.0 ± 0.2	124.1 ± 0.7	0.07	–	–	–
dPG-13Arg13His	10.9 ± 0.8	97.17 ± 0.87	0.13	13	13	1:1
dPG-13Arg	10.6 ± 0.9	60.04 ± 1.2	0.18	13	–	–
dPG-13His	10.3 ± 0.3	70.23 ± 0.8	0.17	–	13	–
dPG-8Arg30His	11.0 ± 0.9	104.9 ± 0.45	0.18	8	30	~1:3

[a]ζ were measured at pH 7.4; [b]intensity distributions are reported; [c]PDI of polyplexes were determined by DLS; [d]degree of functionalization on dPG which were determined by ^{1}H NMR spectroscopy.

with AAdPGs. As shown in Figure 1, all AAdPGs were able to neutralize the negative charge of the siRNA and effectively retard it at N/P ratios between 2 to 4. The binding capacity of all vectors was slightly different from each other. The results of this assay clearly display that all synthesized vectors were able to form polyplexes with siRNA at low N/P ratios. Moreover, the complex formation ability of the new vectors is comparable with dPG-NH_2 50% and 90%.

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

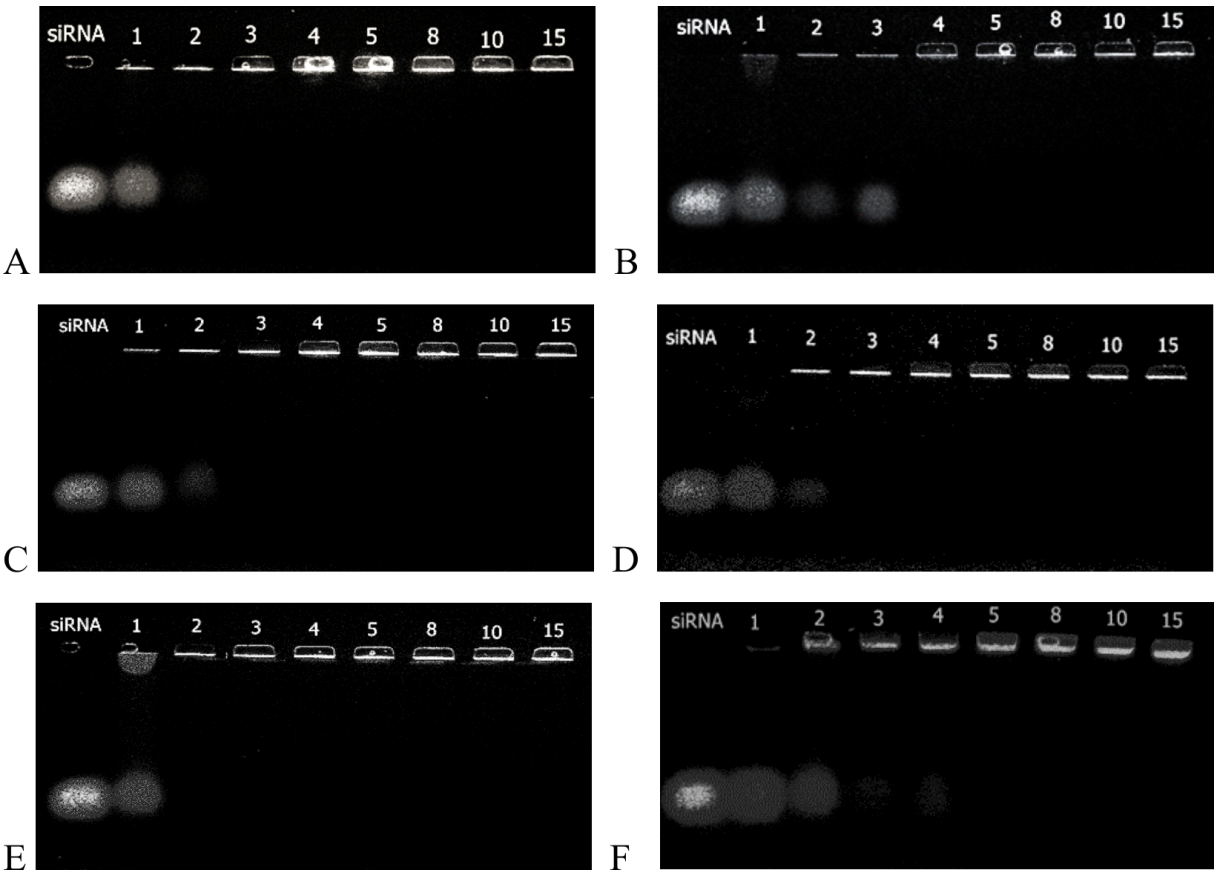


Figure 1: Agarose gel electrophoresis retardation assay of AAdPGs/siRNA polyplexes. (A) dPG-13Arg13His, (B) dPG-13Arg, (C) dPG-13His, (D) dPG-8Arg30His, (E) dPG-NH_2 50%, and (F) dPG-NH_2 90%. Naked siRNA always appears in the first lane. The numbers on the top of each lane correspond to the different N/P ratios.

Average particle size and surface charges of AAdPG/siRNA polyplexes

The appropriate particle size and surface charge are critical characteristics of nanoplexes for efficient transfection [29]. Physicochemical characterization of AAdPG/siRNA polyplexes was conducted using dynamic light scattering (DLS). Figure 2 shows the size distribution of dPG polyplexes (at N/P ratio 10). The average size of all nanoparticles ranges from 60–100 nm. In general, the AAdPG/siRNA polyplexes were smaller than the corresponding dPG-NH_2 50%/siRNA polyplexes. Moreover, AAdPG complexes have a broader distribution of the final nanoparticles. The size of dPG-13Arg and dPG-13His complexes was slightly smaller than the other dPG-based vectors. The surface charge of the final nanoparticles was comparable to the corresponding complexes of siRNA and dPG-NH_2 50% with terminal primary amines and about 10 mV. The positive charge of the polyplexes is a further indication of efficient siRNA complexation by AAdPGs. The results for the size and zeta potential measurements of all vectors are summarized in Table 1.

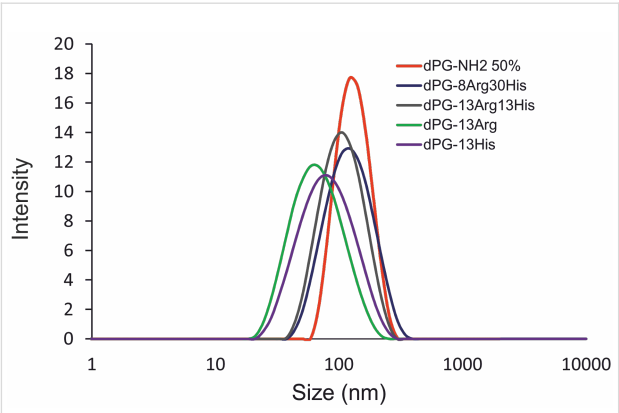


Figure 2: Size measurements of dPG-NH_2 50% and AAdPGs/siRNA complexes. Intensity distributions of all polyplexes are depicted.

Cell viability assay

The cytotoxicity of cationic polymers is mainly attributed to the interactions of these polymers with the cell membrane and depends on multiple factors such as molecular weight, the

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

nature of the polymer surface, and its charge density [30]. The results of the in vitro MTT assays on the NIH 3T3 cell line for cytotoxicity evaluation of AAdPG polyplexes are shown in Figure 3. These results were compared with dPG-NH_2 50% as a backbone and dPG-NH_2 90%. Generally, these data indicates that cytotoxicity of the final polyplexes is reduced by functionalization of dPG-NH_2 50% with Arg and His. Moreover, decreasing the percentage of arginine on a dendritic scaffold improved the cytotoxicity of the nanoplexes. Replacing the primary amines on dPG-NH_2 with histidine groups would possibly decrease the density of positive charge on dPG and increase cell viability. The best cytotoxicity profile was observed for dPG-8Arg30His with no considerable cytotoxicity (cell viability ≥ 90%) up to N/P ratio 40 (Figure 3). We further compared the cytotoxicity of dPG-8Arg30His with dPG-NH_2 90% at N/P ratio 30 where the efficiencies of both vectors were comparable. Overall, these results demonstrated that dPG-8Arg30His is a safer vector compared to dPG-NH_2 90% (Figure 4).

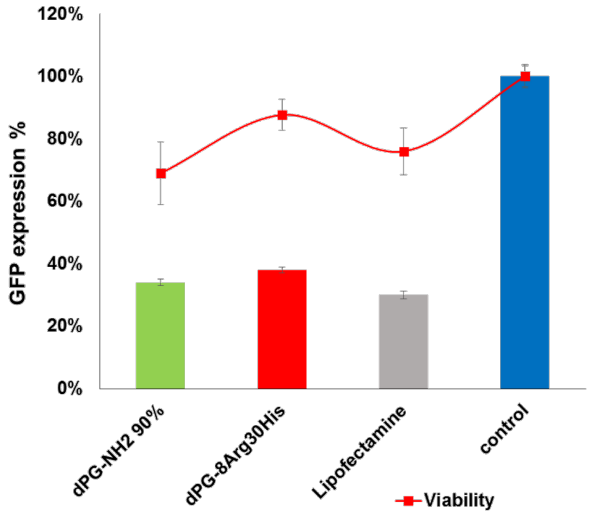


Figure 4: Cell viability versus transfection efficiency of dPG-8Arg30His and dPG-NH_2 90% at N/P ratio 30.

In vitro transfection assay

The transfection efficiency of the AAdPGs was assessed in GFP expressing NIH 3T3 cells (Figure 5). In general, the results indicate that post-modification of the dendritic scaffold with Arg and His improves the efficiency of siRNA transfection. The most efficient vector in the knockdown of GFP (down regulation of GFP expression to 38%) was obtained by converting almost all primary amines on dPG to Arg and His with a 1:3 ratio. Moreover, by comparing the knockdown efficiency of dPG-13Arg (without any histidine functionality) with all the other vectors containing histidine, the critical role of histidine as a buffering agent in enhancing transfection efficiency was determined. Furthermore, we compared the result of our best vector, dPG-8Arg30His, in terms of transfection with dPG-NH_2 90%. These results indicate that dPG-8Arg30His (at N/P ratio 30) is as potent as dPG-NH_2 90% in GFP knockdown while maintaining its low cytotoxicity (Figure 4).

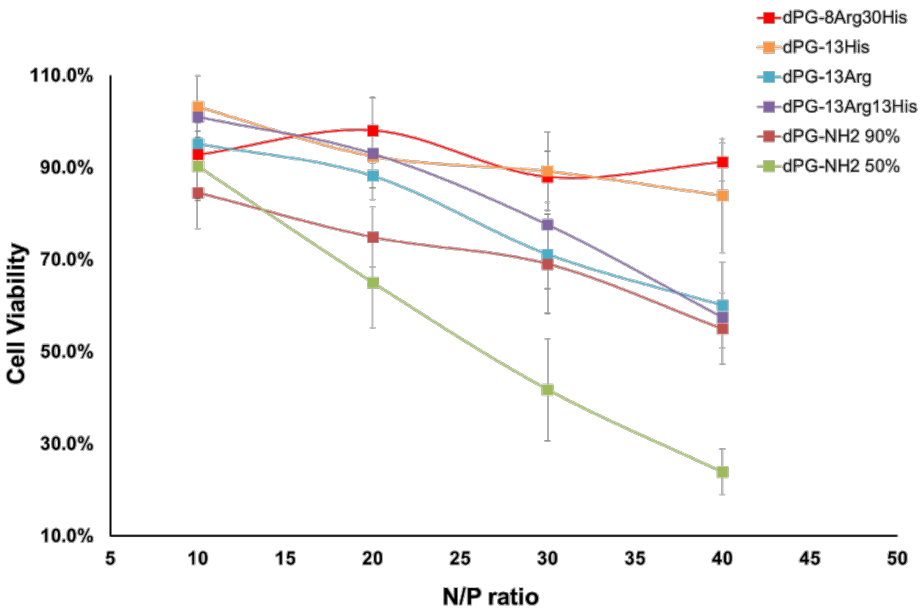


Figure 3: The result of MTT assay on a NIH 3T3 cell line transfected with AAdPG, dPG-NH_2 50%, and 90%/siRNA polyplexes at different N/P ratios with 100 nM siRNA concentration.

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

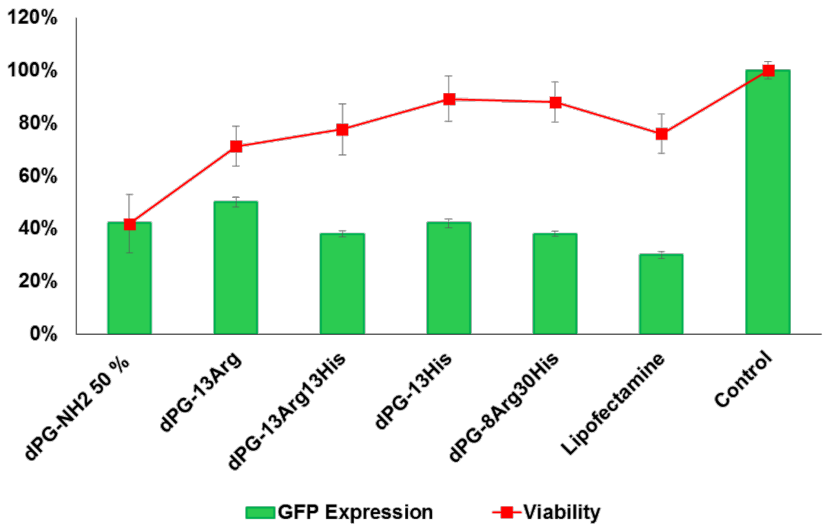


Figure 5: Summary of transfection results versus viability of AAdPGs with various Arg and His composition ratio at N/P ratio 30.

Cellular uptake and confocal microscopy

The cellular uptake and localization of fluorescently labeled siRNA/AAdPG complexes were quantified using flow cytometry and confocal microscopy (Figure 6). By comparing the cellular uptake of dPG-NH_2 functionalized solely with either histidine or arginine, for example, dPG-13Arg, one can clearly see that Arg functionalization improved cellular uptake of both dPG-NH_2s. These results are in agreement with several studies where the transmembrane function of arginine-rich peptides was demonstrated [31,32]. Interestingly, there is a reverse effect with respect to cellular uptake after functionalization of dPG-NH_2 with histidine. Notably, dPG-NH_2s have shown a higher cellular uptake than lipofectamine which is most probably due to their high positive surface charge. These results in combination with transfection efficiency data suggest that the higher transfection efficiency of histidine-functionalized vectors is presumably due to their improved endosomal release.

Conclusion

We successfully post-modified dPG-NH_2 with variable ratios of Arg and His as mimicry of natural histones to afford safe and efficient siRNA transfection. At certain ratios of Arg to His (1:3) a multivalent cationic vector was obtained with comparable transfection efficiency to lipofectamine (down regulation of GFP expression to 37% at N/P ratio 40) and marginal cytotoxicity (cell viability ≥ 90% at N/P ratio 40). The efficiency of this new vector is comparable to our well-studied vector, dPG-NH_2 90%. Post modification of dPG-NH_2 with Arg and His did not dramatically affect the physicochemical properties (particle size and zeta potential) of the resulting vectors and their nanoplexes but notably improved cell viability. This can be attributed to the steric congestion around the amine groups and more biocompatible surface functionalities after amino acid functionalization of dPG-NH_2. Compared to arginine, the introduction of histidine more effectively reduced the cytotoxicity and mediated an efficient endosomal escape. Moreover, by comparing the result of cellular uptake with transfection efficiencies, one can conclude that enhanced cellular uptake does not guarantee by itself efficient siRNA transfection and that incorporation of endosomal releasing groups like histidine seems to play a more crucial role in efficient transfection as compared to arginine.

Experimental

Materials

All chemicals and reagents were used as received from the suppliers without further purification. Protected amino acids and coupling reagents were purchased from abcr GmbH (Karlsruhe, Germany). GelRed™ siRNA stain was purchased from VWR (Radnor, PA). All cell culture media and fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). All siRNA used in this study was purchased from Ambion (Carlsbad, CA) with Silencer® Select negative control siRNA and Silencer®Cy™-3 labeled Negative Control siRNA used for control and cellular uptake studies, respectively. Unmodified Silencer® series siRNA was used for GFP silencing experiments with the following sequence: sense 5'-CAAGCUGACCCUGAAGUUCTT-3' and antisense 5'-GAACUUCAGGGUCAGCUUGCC-3'. All water used in the biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA). Both unmodified and engineered NIH 3T3 cells expressing green fluorescence protein (GFP) were kindly provided by Professor Young Jik Kwon (Department of Chemical Engineering, UC Irvine, CA).

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

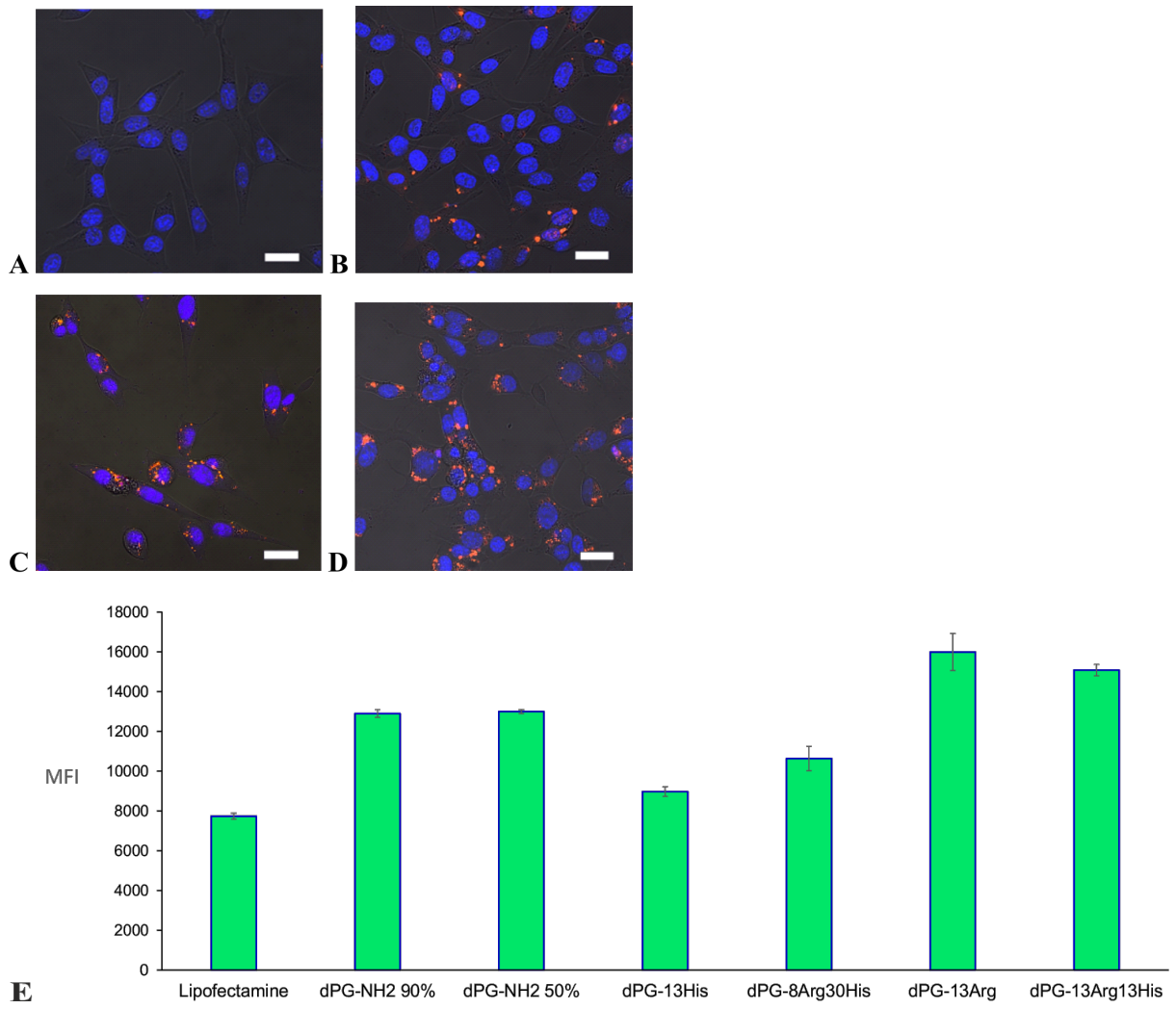


Figure 6: Confocal images of NIH 3T3 cells treated with Cy3-siRNA/vector complexes: (A) naked siRNA, (B) lipofectamine, (C) dPG-13His, (D) dPG-13Arg, and (E) mean Cy-3 fluorescence intensity of 3T3 cells treated with Cy3-siRNA/vector complexes assessed by FACS.

Functionalization of dPG-NH_2 with arginine (Arg) and histidine (His)

dPG (M_n = 8.4 kDa, PDI = 1.7) was prepared according to a published procedure [33]. Fifty percent of all (~110) hydroxy groups on dendritic polyglycerol were functionalized with amino groups in a three-step protocol [27]. Briefly, the transformation was started with the mesylation of the hydroxy groups on dPG. In the next step, the mesylated polyglycerol was converted to polyglycerolazide. In the last step, azide functionalities (N_3) were reduced to primary amines (-NH_2) via Staudinger reduction (Scheme S1 in Supporting Information File 1). For coupling both amino acids Arg and His to the dendritic backbone, a solution of dPG-NH_2, 30 mg (0.20 mmol of amines) in methanol, was dried carefully under high vacuum. The concentrated solution was then diluted in 1.5 mL DMSO. The solution of dPG-NH_2 in DMSO was left under vacuum for 30 min in order to remove methanol residues. Boc-protected histidine and arginine were added to the solution of dPG-NH_2 in specific molar ratios. 1.2 Equivalents of BOP and DIPEA with respect to the amino groups were added to the reaction subsequently. The reaction mixture was stirred at room temperature overnight. This mixture was then transferred directly into a dialysis tube of 1000 MWCO and dialyzed in methanol for 2 days. After removing methanol on a rotary evaporator completely, the reaction mixture was treated with a mixture of TFA/DCM/TIPS. The reaction was left running overnight to complete the deprotection. After the deprotection step, dialysis in 0.2 N solution of HCl for two days resulted in the formation of products as chloride salt which were obtained as pale yellow to brown solids by lyophilization. Noteworthy that each dPG unit (10 kDa) has is about 100 hydroxy groups and therefore the functionalization percentages always corresponds approxi-

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

mately to the same number of functional groups per dPG. For example, dPG-NH_2 50% has about 50 NH_2 groups per polymer unit. The amino acid functionalization percentage of each polymer was defined using ^{1}H NMR analysis. ^{1}H NMR (400 MHz, D_2O) dPG-13Arg13His: δ = 1.6 (s, $NHCH_2CH_2CH_2CH$, 2H), 1.9 (s, $NHCH_2CH_2CH_2CH$, 2H), 3–4.5 (m, dPG backbone, $NHCH_2CH_2CH_2CH$ and $NHCH_2CH_2CH_2CHNH_2CO$ of arginine groups, $NH_2COCHCH_2C$ and $NH_2COCHCH_2C$ of histidine groups), 7.4 (s, C*H*NHCHN, 1H of imidazole groups) and 8.7 (s, CHNHC*H*N, 1H of imidazole groups) ppm. dPG-13Arg: δ = 1.6 (s, $NHCH_2CH_2CH_2CH$, 2H), 1.9 (s, $NHCH_2CH_2CH_2CH$, 2H), 3–4.5 (m, dPG backbone, $NHCH_2CH_2CH_2CH$ and $NHCH_2CH_2CH_2CHNH_2CO$ of arginine groups) ppm. dPG-13His: δ = 3–4.5 (m, dPG backbone, $NH_2COCHCH_2C$ and $NH_2COCHCH_2C$ of histidine groups), 7.4 (s, C*H*NHCHN, 1H of imidazole groups) and 8.7 (s, CHNHC*H*N, 1H of imidazole groups) ppm. dPG-8Arg30His: δ = 1.6 (s, $NHCH_2CH_2CH_2CH$, 2H), 1.9 (s, $NHCH_2CH_2CH_2CH$, 2H), 3–4.5 (m, dPG backbone, $NHCH_2CH_2CH_2CH$ and $NHCH_2CH_2CH_2CHNH_2CO$ of arginine groups, $NH_2COCHCH_2C$ and $NH_2COCHCH_2C$ of histidine groups), 7.4 (s, C*H*NHCHN, 1H of imidazole groups) and 8.7 (s, CHNHC*H*N, 1H of imidazole groups) ppm.

Gel electrophoresis

The binding of AAdPGs to siRNA was evaluated by agarose gel electrophoresis retardation assay. Stock solutions of siRNA and AAdPGs were prepared in phosphate buffer (10 mM, pH 7.4). To a 2 μL solution of siRNA (4 μM), different amounts of AAdPG compounds were added to achieve different N/P ratios (the molar ratio between amine groups of dPGs to siRNA phosphate groups). The final volume of the mixture was adjusted to 12.5 μL by the same buffer solution. siRNA and AAdPGs were incubated at room temperature for 30 min. After incubation, 2.5 μL of 6X orange gel loading dye was added to each sample. 10 μL of the final mixture was then loaded on a 1% agarose gel with 1X GelRed™. After filling the gel packets with polyplexes, electrophoresis was run in TAE buffer for 45 min at 60 V. The results were visualized under UV illumination.

DLS/Zeta

The size and zeta potential (ζ) of AAdPG/siRNA polyplexes were measured by a Zetasizer Nano ZS analyzer™ with integrated 4 mW He-Ne laser, λ = 633 nm (Malvern Instruments™ Ltd, U.K.). Stock solutions of dPG samples and siRNA (50 μM) in nanopure water were prepared. An appropriate amount of each dPG sample was mixed with 2.85 μL siRNA (6 nmol phosphate) solution. The mixtures were diluted to 100 μL and after short vortexing were incubated for 30 min at rt. Subsequently, DLS measurements were recorded. The same mixture from DLS measurements was taken and diluted with 0.8 μL phosphate buffer (10 mM, pH 7.4). These samples were then subjected to zeta potential measurements. The measurements were repeated at least three times for each sample and the mean values were reported.

MTT assay

Unmodified NIH 3T3 cells were seeded at a density of 5,000 cells per well in 96-well plates 24 h in advance. The culture media was changed from 100 μL DMEM with 10% fetal bovine serum (FBS) to 80 μL plain DMEM immediately before exposure to the complexes. The dPG/siRNA complexes were prepared by first diluting the siRNA to 1.5 μM with PBS (10 mM phosphate, 10 mM NaCl, pH 7.4) and then adding the proper amount of vector solution (5 mg/mL in ddH_2O) to give the desired N/P ratio and concentration. After 30 minutes incubation at rt, 20 μL of the complex solutions were added to each well to give a final volume of 100 μL per well. After 4 h incubation, the media was replaced with 10% FBS/DMEM and the cells cultured for another 48 h. To assess the viability, the media was replaced with 50 μL DMEM solution containing 0.5 mg/mL MTT, followed by 4 h incubation at 37 °C. 100 μL of DMSO was added to each well to dissolve the formazan and the plate was incubated at 37 °C for 30 min with agitation. The absorbance at 540 nm was measured using a plate reader and the viability determined by comparison with untreated controls.

Transfection

NIH 3T3 fibroblast cells expressing GFP were seeded at a density of 10,000 cells/well in 48-well plates 24 h in advance and the culture media replaced with 200 μL plain DMEM immediately prior to transfection. AAdPG/siRNA complexes were prepared as described previously with either anti-GFP siRNA or negative control siRNA. 50 μL of the complex solutions were added to each well to give a final volume of 250 μL per well. After 4 h incubation, the media was replaced with 10% FBS/DMEM and the cells cultured for another 48 h. Before the analysis, cells were released from each well with trypsin and harvested by centrifugation (5 min, 500G). GFP fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser. For each sample, data representing 10,000 objects were collected as a list-mode file and analyzed using FACSDivaTM software (Becton Dickinson, version 6.1.3) and the percent knockdown was calculated by comparing the mean fluorescence intensity of cells treated with vector/anti-GFP siRNA to that of cells treated with complexes formed with the control siRNA.

Cellular uptake study

For quantitative assessment of cellular uptake, negative control siRNA labeled with Cy3 (siRNA-Cy3) was complexed with the

Beilstein J. Org. Chem. **2015,** *11,* 763–772.

vectors in PBS as described previously. Unmodified NIH 3T3 cells were seeded in 48-well plates and transfected with the siRNA-Cy3/vector complexes following the same transfection protocol used for GFP silencing experiments. Immediately after the 4 h exposure to the transfection media, the cells were trypsinized and collected via centrifugation. The transfected cells were analyzed by FACS to determine the mean Cy3-fluoroscence of each sample.

Confocal microscopy

Unmodified NIH 3T3 fibroblast cells were seeded at a density of 10,000 cells/well on an 8-well chamber slide (Lab-Tek, Rochester, NY) 24 h before transfection. Cy3-labeled siRNA was complexed with the vectors and the cells transfected with the complexes following the previously described protocol. After 4 h exposure to the transfection media, the media was changed back to DMEM supplemented with 10% fetal bovine serum. Confocal fluorescence spectroscopy was performed at different time points after the transfection. The nuclei were stained with Hoechst 33342 following the standard protocol. The images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope with a 40× numerical aperture oil immersion planapochromat objective. A 559 nm helium–neon laser, a SMD640 dichroic mirror, and a 575–620 nm band-pass barrier filter were used to obtain the images of Cy3-labeled siRNA. Images of the stained nuclei were acquired using a 780 nm two-photon excitation light, a 635 nm dichroic mirror, and a 655–755 nm band-pass barrier filter. The two fluorescent images were scanned separately and overlaid together with the differential interference contrast image (DIC). The cells were scanned as a z-stack of two-dimensional images (1024 × 1024 pixels) and an image cutting approximately through the middle of the cellular height was selected to present the intracellular siRNA localization.

Statistical analysis

All transfection studies were performed in triplicates; data were expressed as mean ± SEM.

Supporting Information

Supporting Information File 1

Synthetic procedure of dPG-NH_2 and NMR spectra.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-11-86-S1.pdf]

Acknowledgements

FSM would like to thank SFB 765 and BMBF (Bundesministerium für Bildung und Forschung) for financial support. We would like to thank Dr. Pamela Winchester for proofreading of the manuscript. ZG would like to acknowledge the financial support of the US National Institute of Health (DK098446).

References

1. Kanasty, R.; Dorkin, J. R.; Vegas, A.; Anderson, D. *Nat. Mater.* **2013,** *12,* 967–977. doi:10.1038/nmat3765
2. DiFiglia, M.; Sena-Esteves, M.; Chase, K.; Sapp, E.; Pfister, E.; Sass, M.; Yoder, J.; Reeves, P.; Pandey, R. K.; Rajeev, K. G.; Manoharan, M.; Sah, D. W. Y.; Zamore, P. D.; Aronin, N. *Proc. Natl. Acad. Sci. U. S. A.* **2007,** *104,* 17204–17209. doi:10.1073/pnas.0708285104
3. Whitehead, K. A.; Langer, R.; Anderson, D. G. *Nat. Rev. Drug Discovery* **2009,** *8,* 129–138. doi:10.1038/nrd2742
4. Mehrabadi, F. S.; Fischer, W.; Haag, R. *Curr. Opin. Solid State Mater. Sci.* **2012,** *16,* 310–322. doi:10.1016/j.cossms.2013.01.003
5. Sunder, A.; Hanselmann, R.; Frey, H.; Mülhaupt, R. *Macromolecules* **1999,** *32,* 4240–4246. doi:10.1021/ma990090w
6. Calderón, M.; Quadir, M. A.; Sharma, S. K.; Haag, R. *Adv. Mater.* **2010,** *22,* 190–218. doi:10.1002/adma.200902144
7. Kainthan, R. K.; Janzen, J.; Levin, E.; Devine, D. V.; Brooks, D. E. *Biomacromolecules* **2006,** *7,* 703–709. doi:10.1021/bm0504882
8. Kainthan, R. K.; Brooks, D. E. *Biomaterials* **2007,** *28,* 4779–4787. doi:10.1016/j.biomaterials.2007.07.046
9. Wei, Q.; Krysiak, S.; Achazi, K.; Becherer, T.; Noeske, P.-L. M.; Paulus, F.; Liebe, H.; Grunwald, I.; Dernedde, J.; Hartwig, A.; Hugel, T.; Haag, R. *Colloids Surf., B* **2014,** *122,* 684–692. doi:10.1016/j.colsurfb.2014.08.001
10. Gröger, D.; Paulus, F.; Licha, K.; Welker, P.; Weinhart, M.; Holzhausen, C.; Mundhenk, L.; Gruber, A. D.; Abram, U.; Haag, R. *Bioconjugate Chem.* **2013,** *24,* 1507–1514. doi:10.1021/bc400047f
11. Calderón, M.; Welker, P.; Licha, K.; Fichtner, I.; Graeser, R.; Haag, R.; Kratz, F. *J. Controlled Release* **2011,** *151,* 295–301. doi:10.1016/j.jconrel.2011.01.017
12. Hussain, A. F.; Krüger, H. R.; Kampmeier, F.; Weissbach, T.; Licha, K.; Kratz, F.; Haag, R.; Calderón, M.; Barth, S. *Biomacromolecules* **2013,** *14,* 2510–2520. doi:10.1021/bm400410e
13. Kim, T. H.; Ihm, J. E.; Choi, Y. J.; Nah, J. W.; Cho, C. S. *J. Controlled Release* **2003,** *93,* 389–402. doi:10.1016/j.jconrel.2003.08.017
14. Park, S.; Lee, S. K.; Lee, K. Y. *J. Controlled Release* **2011,** *152,* e165–e166. doi:10.1016/j.jconrel.2011.08.065
15. Noh, S. M.; Park, M. O.; Shim, G.; Han, S. E.; Lee, H. Y.; Huh, J. H.; Kim, M. S.; Choi, J. J.; Kim, K.; Kwon, I. C.; Kim, J.-S.; Baek, K.-H.; Oh, Y.-K. *J. Controlled Release* **2010,** *145,* 159–164. doi:10.1016/j.jconrel.2010.04.005
16. Swami, A.; Aggarwal, A.; Pathak, A.; Patnaik, S.; Kumar, P.; Singh, Y.; Gupta, K. C. *Int. J. Pharm.* **2007,** *335,* 180–192. doi:10.1016/j.ijpharm.2006.11.033
17. Kim, T.-i.; Baek, J.-u.; Yoon, J. K.; Choi, J. S.; Kim, K.; Park, J.-s. *Bioconjugate Chem.* **2007,** *18,* 309–317. doi:10.1021/bc0601525
18. Midoux, P.; Pichon, C.; Yaouanc, J. J.; Jaffres, P.-A. *Br. J. Pharmacol.* **2009,** *157,* 166–178. doi:10.1111/j.1476-5381.2009.00288.x
19. Choi, J. S.; Nam, K.; Park, J.-y.; Kim, J.-B.; Lee, J.-K.; Park, J.-s. *J. Controlled Release* **2004,** *99,* 445–456. doi:10.1016/j.jconrel.2004.07.027
20. Kim, T.-i.; Baek, J.-u.; Bai, C. Z.; Park, J.-s. *Biomaterials* **2007,** *28,* 2061–2067. doi:10.1016/j.biomaterials.2006.12.013

21. Luger, K.; Mäder, A. W.; Richmond, R. K.; Sargent, D. F.; Richmond, T. J. *Nature* **1997,** *389,* 251–260. doi:10.1038/38444
22. Ofek, P.; Fischer, W.; Calderón, M.; Haag, R.; Satchi-Fainaro, R. *FASEB J.* **2010,** *24,* 3122–3134. doi:10.1096/fj.09-149641
23. Fischer, W.; Calderón, M.; Schulz, A.; Andreou, I.; Weber, M.; Haag, R. *Bioconjugate Chem.* **2010,** *21,* 1744–1752. doi:10.1021/bc900459n
24. Luo, K.; Li, C.; Li, L.; She, W.; Wang, G.; Gu, Z. *Biomaterials* **2012,** *33,* 4917–4927. doi:10.1016/j.biomaterials.2012.03.030
25. Zeng, H.; Little, H. C.; Tiambeng, T. N.; Williams, G. A.; Guan, Z. *J. Am. Chem. Soc.* **2013,** *135,* 4962–4965. doi:10.1021/ja400986u
26. Kasai, S.; Nagasawa, H.; Shimamura, M.; Uto, Y.; Hori, H. *Bioorg. Med. Chem. Lett.* **2002,** *12,* 951–954. doi:10.1016/S0960-894X(02)00066-5
27. Roller, S.; Zhou, H.; Haag, R. *Mol. Diversity* **2005,** *9,* 305–316. doi:10.1007/s11030-005-8117-y
28. Mitchell, D. J.; Steinman, L.; Kim, D. T.; Fathman, C. G.; Rothbard, J. B. *J. Pept. Res.* **2000,** *56,* 318–325. doi:10.1034/j.1399-3011.2000.00723.x
29. Ramezani, M.; Malaekeh-Nikouei, B.; Malekzadeh, S.; Baghayeripour, M. R.; Malaekeh-Nikouei, M. *Curr. Nanosci.* **2012,** *8,* 680–684. doi:10.2174/157341312802884535
30. Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. *Biomaterials* **2003,** *24,* 1121–1131. doi:10.1016/S0142-9612(02)00445-3
31. Fischer, P. M.; Krausz, E.; Lane, D. P. *Bioconjugate Chem.* **2001,** *12,* 825–841. doi:10.1021/bc0155115
32. Schwartz, J. J.; Zhang, S. G. *Curr. Opin. Mol. Ther.* **2000,** *2,* 162–167.
33. Haag, R.; Tuerk, H.; Mecking, S. Verfahren zur Herstellung Hochverzweigter Polymere. Ger. Pat. Appl. DE 10211664 A1, Oct 2, 2003.

License and Terms

Supporting information

for

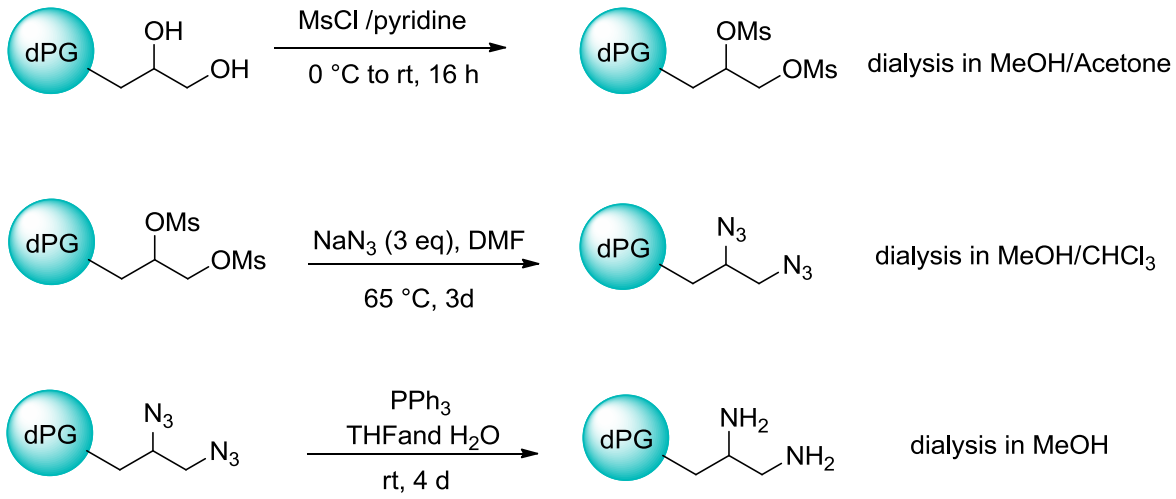
Multivalent dendritic polyglycerolamine with arginine and histidine end groups for efficient siRNA transfection

Fatemeh Sheikhi Mehrabadi[1], Hanxiang Zeng[2], Mark Johnson[2], Cathleen Schlesener[1], Zhibin Guan [*2] and Rainer Haag[*1]

Address: [1]Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany and [2]Department of Chemistry, University of California, 1102 Natural Sciences 2, Irvine, California 92697-2025, USA

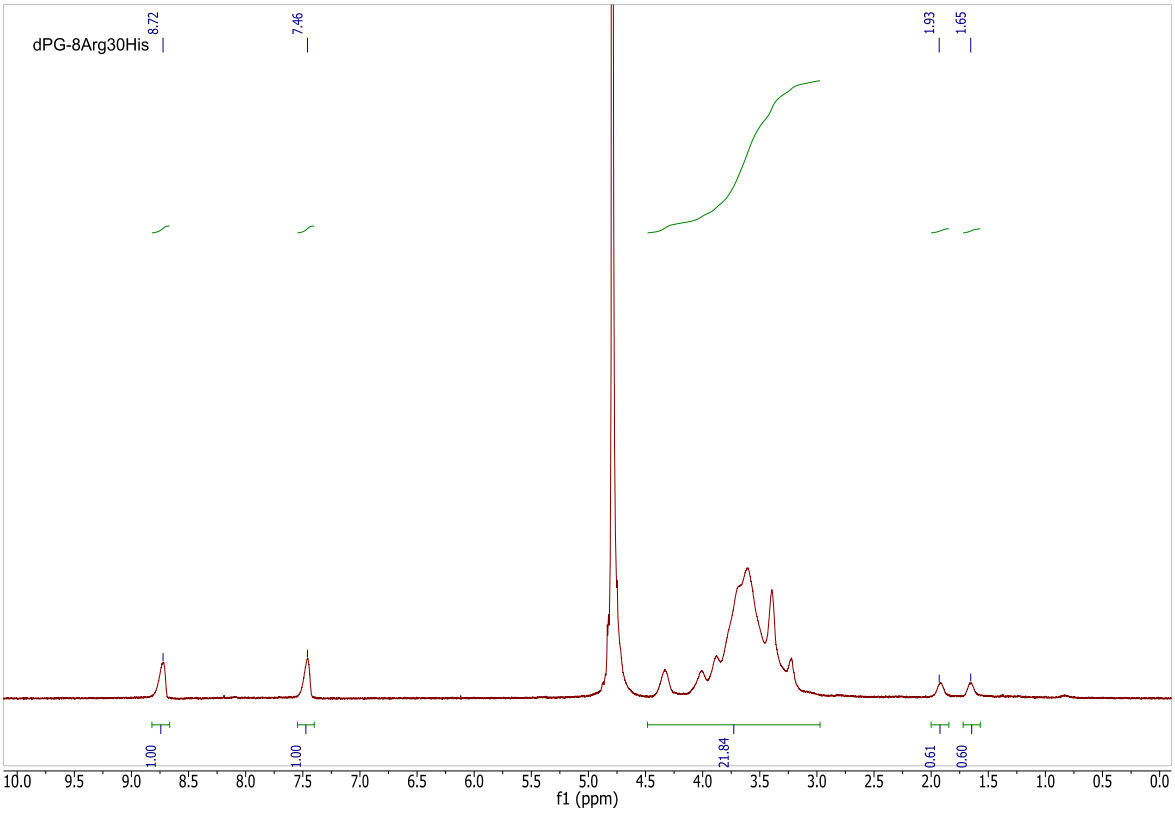
*Corresponding author
Email: Zhibin Guan - zguan@uci.edu; Rainer Haag - haag@chemie.fu-berlin.de

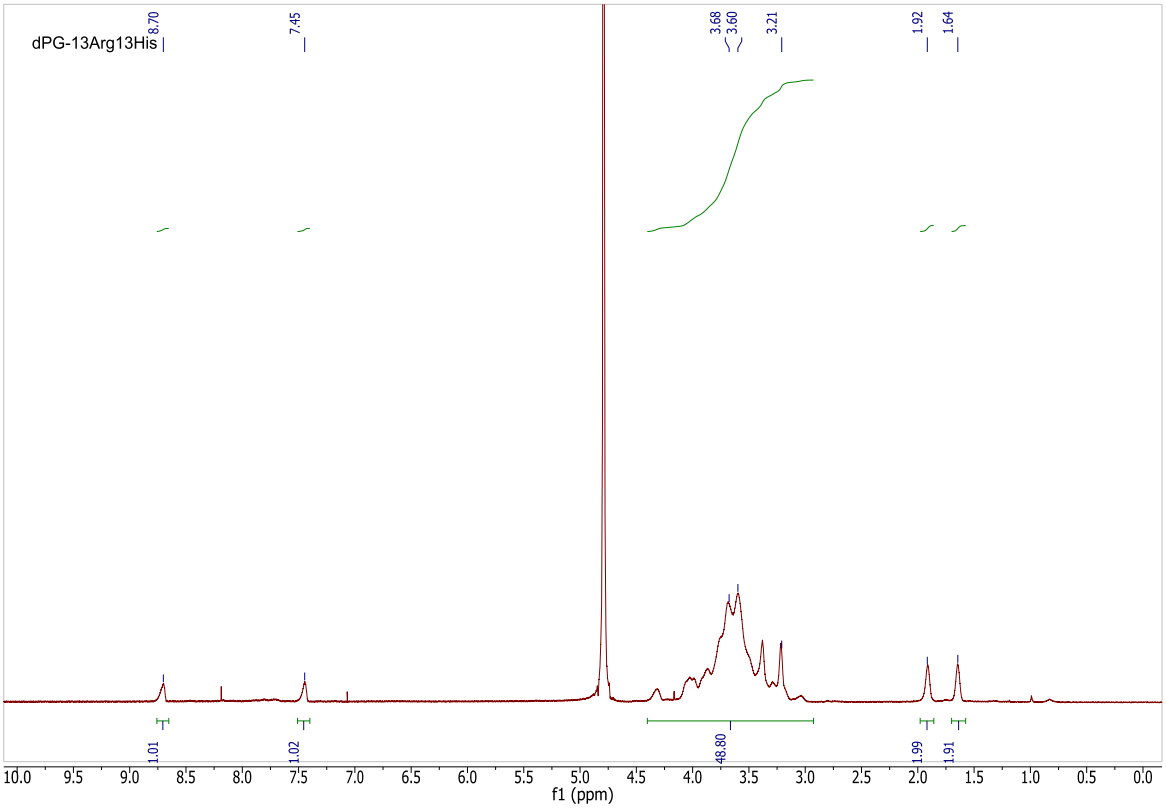
Synthetic procedure of dPG-NH_2 and NMR spectra

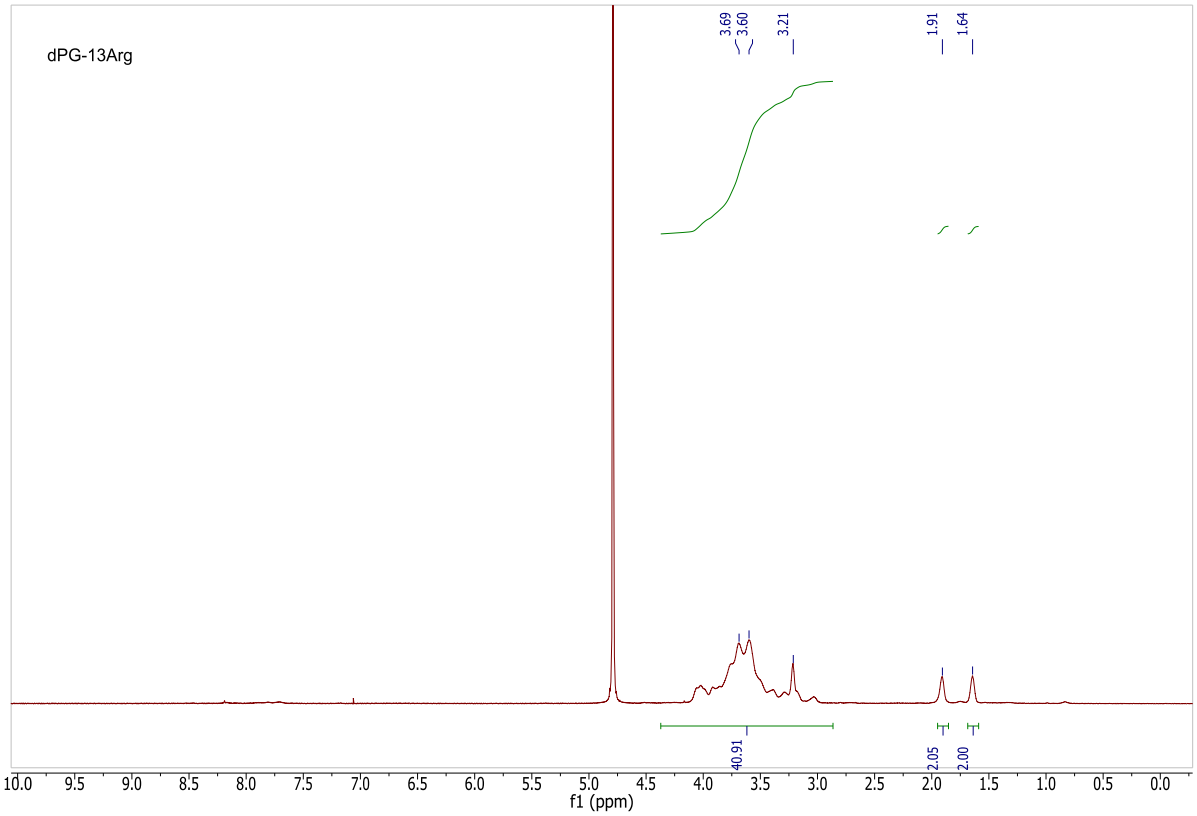


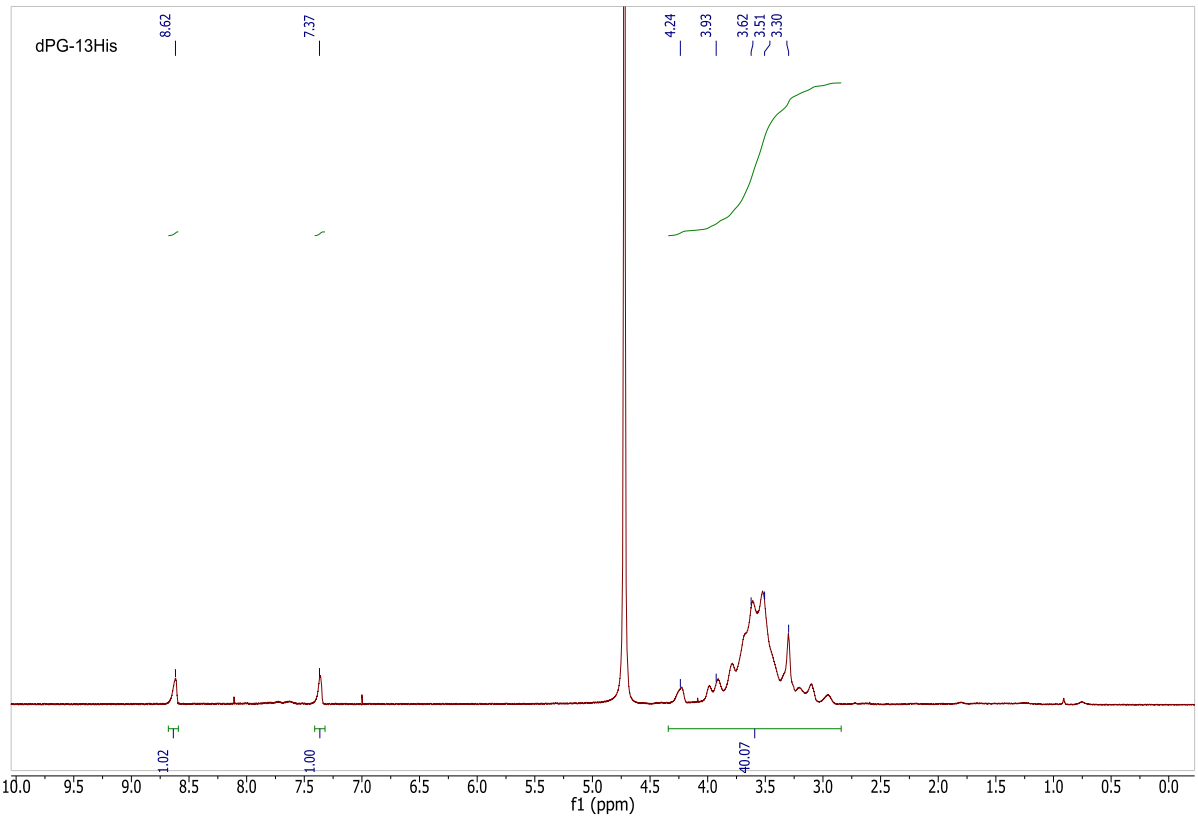
Scheme S1: Three step synthetic procedure of dPG-NH_2.

^{1}H NMR of all synthetic AAdPG vectors:

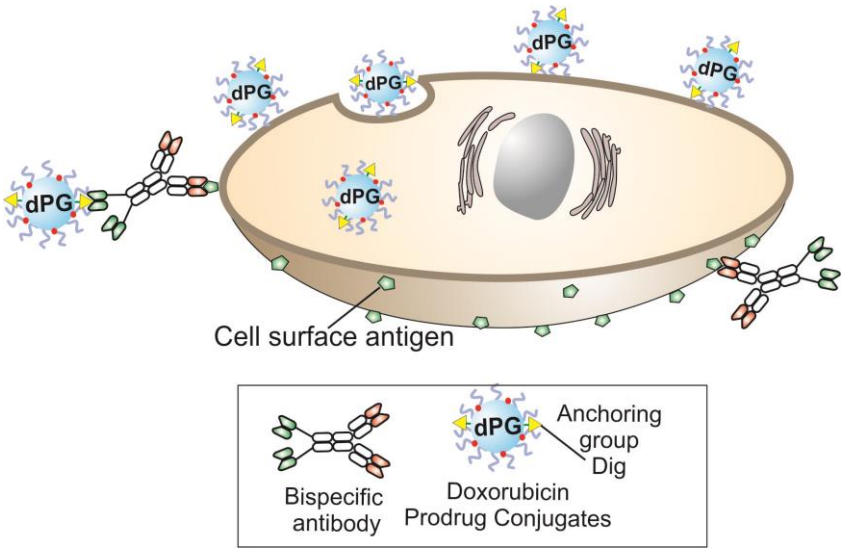





dPG-13Arg
3.69
3.60
3.21
1.91
1.64
40.91
2.05
2.00
f1 (ppm)


dPG-13His
8.62
7.37
4.24
3.93
3.62
3.51
3.30
1.02
1.00
40.07
f1 (ppm)

3.4 Bispecific Antibodies for Targeted Delivery of Dendritic Polyglycerol (dPG) Prodrug Conjugates



This work was published as follows:

Fatemeh Sheikhi Mehrabadi, Juliane Adelmann, Shilpi Gupta, Stefanie Wedepohl, Marcelo Calderón, Ulrich Brinkmann, and Rainer Haag, *Curr Cancer Drug Targets* **2016**, *16*, 1-11.

DOI: 10.2174/1568009616666160208142910

Author's contributions

- Synthesis of dPG-based prodrug conjugates
- Full characterization of dPG-based prodrug conjugates
- Analysis of the data and preparation of the manuscript

4 Summary and Conclusion

Highly amine functionalized dendritic polyglycerol (dPG-NH_2 90%, average MW ~ 10 kDa) has shown promise in siRNA transfection. Nonetheless, toxicity at therapeutic doses is a limiting factor for further development of its potential applications. In the first part of this thesis, dPG analogues with various amine DF (10, 30, and 50%) were synthesized and biologically evaluated to find a compromise between their cytotoxicity and siRNA transfection efficiency. As a result of the *in vitro* and *in vivo* evaluation of these molecules, 50% was a minimum required DF to achieve efficient siRNA transfection. These results were further compared to dPG-NH_2 90%. Unluckily, when 50% of hydroxyl groups on a 10 kDa dPG were substituted with primary amines, the resulting molecule showed similar toxicity to dPG-NH_2 90%. In parallel, the results of theoretical studies by atomistic molecular dynamics simulations on the interactions between dPG-NH_2 analogs, with a 21-bp DNA model predicted a similar trend and emphasized that a minimum of 50% amine DF (on the same dPG core) is necessary to achieve efficient nucleic acid complexation. In the second part of this work, dPG of two different molecular weights (*Mn* = 8 and 43 kDa) with different amine DF (50, 70, and 90%) were synthesized, characterized, and biologically evaluated respecting cytotoxicity and transfection efficiency *in vitro* and *in vivo*. Interestingly, a dPG-NH_2 analogue with number average molecular weight of 43 kDa and 50% DF outperformed the 10 kDa dPG-NH_2 90%. In this study, it was concluded that the higher number of amines on 43 kDa dPG-NH_2 50% rendered more efficient siRNA complexation and thereby more effective transfection. Likewise, the higher molecular weight (therefore higher hydrodynamic diameter) of dPG core resulted in a lower overall charge density so that a safer *in vivo* profile was obtained compared to the dPG-NH_2 90%. Furthermore, the high tolerability of dPG-NH_2 50% (43 kDa) allowed application of higher dosing in the *in vivo* knock down studies that gave rise to highly efficient and specific luciferase gene knock down in tumor bearing mice. In the third part of this work, we explored the effect from introducing different amine types than primary amines to dPG to improve its cytotoxicity. Therefore, primary amines on a 10 kDa dPG-NH_2 50% were post-modification with different ratios of two basic amino acids, namely arginine and histidine. The resulting amino acid functionalized dPG-based nanocarriers (AAdPG) were synthesized and their corresponding polyplexes with siRNA were analyzed regarding physico-chemical characterization, cellular uptake, cytotoxicity, and efficiency of GFP expression knock down. Among the synthesized molecules, an optimal vector (down regulation of GFP expression to 38%) and minimal cytotoxicity (cell viability $\geq$ 90%) was obtained. Interestingly, the

cytotoxicity of dPG-NH_2 50% significantly decreased as a result of amino acid functionalization. More importantly, this study demonstrated that histidine residues play a critical role as buffering agents in improving the transfection efficiency of AAdPG nanocarriers.

In the last part of this work, the feasibility of a new active targeting approach based on bispecific antibodies (bsAbs) for selective delivery of dPG-based prodrug conjugates into MCF- 7 tumor cell line was investigated. In this study, bsAbs simultaneously bind to both cell-surface markers and hapten-functionalized payloads and thus specifically deliver them into tumor tissue. Therefore, dPG-based prodrugs conjugates were prepared that had been functionalized with digoxigenin (a well-known hapten as anchoring moiety), a doxorubicin prodrug, and the shielding moiety (PEG). The complex formation between the digoxigeninylated conjugates and bsAbs was demonstrated *in vitro*. The resulting complexes showed binding to the cell surface of Lewis Y (LeY) expressing MCF-7 cells. Furthermore, flow cytometry indicated the preferential binding and uptake of targeted conjugate into MCF-7 cells. Additionally, the digoxigeninylated conjugates showed an increased targeted cytotoxicity in a small window and short incubation time. However, the cytotoxicity of targeting and non-targeting conjugates was similar and independent of bsAb's presence at relevant concentrations for drug delivery. Overall, this study showed the potential of bsAbs as a new targeting approach for tumor specific drug delivery. Nevertheless, the result of cytotoxicity assay revealed that in the design of nanocarriers, special attention should be paid to issues like minimizing non-specific cell adhesion and uptake to achieve maximum targeted delivery results.

The results of this work advance our knowledge on the design and synthesis of more efficient dPG-based drugs, especially siRNA nanocarriers. In fact, this work defines the directions to adapt the dPG scaffold in such a way to obtain more efficient and less cytotoxic siRNA nanocarriers. As a conclusion, when designing dPG-based drug nanocarriers, a great deal of attention should be paid to the choice of molecular weight, DF, and type of functional groups, i.e, amine groups for genetic material delivery. Furthermore, it is concluded that dPG of higher MW offer stronger complexation of siRNA at low N/P ratios and safer toxicity profile presumably due to their lower charge density. Introducing other amine functionalities rather than primary amines, to the dPG scaffold particularly groups that promote endosomal release properties, are highly preferred for safe and efficient transfection. Finally, bsAbs may be *adapted* as an attractive targeting strategy to deliver siRNA into tumor tissue selectively.

5 Outlook

In future work, by combining the results of structure-activity relationship studies, synthesis of amino acid functionalized dPG amine analogues of high molecular weight (~ 43 kDa) with a DF of 30 to 40%, and about 1:3 ratio of arginine to histidine may result in more efficient and safer siRNA nanocarriers. Furthermore, employment of bispecific antibodies (bsAbs) for tissues selective delivery of siRNA therapeutics might be advantageous. Other targeting approaches like grafting receptor ligands on the surface of dPG-NH_2 could be a straightforward approach to achieve targeted nucleic acid delivery. Synthesis and evaluation of highly amine functionalized dPG analogues decorated with peptides that target specific receptors would be of interest.

Another further direction can perhaps be followed to obtain effective transfection with low cytotoxicity using dPG-based carriers by masking the surface charge of highly amine functionalized dPG molecules. In this regard, special attention should be paid to the nature of introduced groups and their degree of functionalization on dPG. Moreover, functionalization of the primary amine on the surface of dPG with charge reversal groups, which can "switch" from negative to positive charge upon stimuli like changes in environmental pH,[261] may improve the toxicity associated with highly amine functionalized dPG molecules.

6 Abstract and Zusammenfassung

6.1 Abstract

Drug delivery systems are necessary to overcome multiple extracellular and intracellular barriers that are ahead of conventional low molecular weight and macromolecular drugs like siRNA. Cytosolic delivery of the siRNA results in highly specific silencing of diseases-associated mRNAs and thereby addressing many diseases at the molecular level. Dendritic polyamines possess multiple favorable characteristics for nucleic acid delivery including facile complex formation with a negatively charged backbone of DNA/siRNA through multiple amine functionalities, mediation in cellular uptake, and endosomal release and the structural features like flexibility that play a crucial role in efficient transfection.

We have already developed a highly functionalized dPG-based polyamine (dPG-NH_2 with 90% amine degree of functionalization (DF) and MW ~ 10 kDa) for siRNA transfection both *in vitro* and *in vivo*. However, the therapeutic window of this vector is too small that restricts its *in vivo* applications. Therefore, a major part of this Ph.D. thesis has focused on altering multiple structural features such as DF, the nature of the introduced amine groups, and dPG's core size to acquire more efficient and safer siRNA delivery using dPG-NH_2 analogues.

First, in a structure-activity relationship study, we synthesized and evaluated dPG-NH_2 analogues of various amine DFs (on dPG MW ~ 10 kDa) for siRNA complexation, cytotoxicity, and knockdown efficiency *in vitro* and *in vivo*. As a results, it was found that certain DF (i.e. 50% on a 10 kDa dPG) is necessary to achieve efficient siRNA transfection. This results was further confirmed by theoretical studies that predicted stronger interactions between dPG-NH_2 analogues of 50% and higher DF with a 21-base pair DNA model.

In the second part of this work, we functionalized dPG of high molecular weight (dPG *Mn* ~ 43 kDa) with amines of different DF to alter the ultimate safety profile of dPG-NH_2 *in vivo*. The results of *in vivo* studies using dPG-NH_2 50% (dPG *Mn* ~ 43 kDa) demonstrated much lower induced immune responses and higher luciferase knockdown efficiency compared to its analogues of lower molecular weight (dPG MW ~10 kDa). Therefore, an effective siRNA vector with optimal charge density was achieved which possess a high enough positive charge to promote cellular uptake and complex formation of siRNA at low N/P ratios but still has a low enough charge to avoid inducing immune responses.

We further introduced arginine and histidine to the primary amines on the periphery of dPG (MW 10 kDa) to retain the effectiveness of siRNA transfection by dPG-NH_2 while improving its cytotoxicity. As a results, an optimal siRNA vector with comparable transfection efficiency to Lipofectamine® and minimal cytotoxicity was obtained. Interestingly, introducing these amino acids improved both the *in vitro* transfection efficiency and cytotoxicity of dPG-NH_2.

Finally, bispecific antibodies (bsAbs) with dual specificity were recruited for delivery of dPG-based prodrug conjuagtes functionalized with a hapten called digoxigenin, into MCF-7 cells expressing LeY antigens. In this study, the targeted binding and internalization of dPG prodrug conjugates was demonstrated. However, no target specific toxicity was observed for digoxigenin functionalized dPG prodrug conjuagtes that might be attributed to the non-specific interaction and uptake of prodrug conjugates that strongly compete with active route of delivery.

All the studies that have been presented in this work emphasize on a diligent and tailor-made design of dPG-based delivery systems according to their final application. Hence, certain amine DFs are necessary to obtain efficient siRNA transfection *in vitro* and *in vivo*. Furthermore, dPG-NH_2 of higher molecular weight and lower DF compared to its analogue of lower molecular weight shows superior siRNA transfection and safer toxicity profile *in vivo*. Additionally, the nature of amine groups as siRNA complexing agents and the presence of groups that promote endosomal release properties are likely key elements for efficient and safe siRNA transfection. Last but not least, active targeting strategies like application of bsAbs can be *adapted* for tissue specific siRNA delivery.

6.2 Zusammenfassung

Die Verabreichung von nackten Wirkstoffen ist mit zahlreichen intra- und extrazellulären Barrieren verbunden, die auf die pharmakokinetischen Eigenschaften des jeweiligen Wirkstoffes, wie beispielsweise Doxorubicin oder siRNA zurückzuführen sind. Um diese Hürden zu überwinden sind Wirkstoffträgersysteme notwendig, die in der Lage sind diese Wirkstoffe sicher zu verkapseln und auf ihrem Weg von der Verabreichung bis zum Wirkort zu schützen und schließlich freizusetzen. Durch diesen Transport kann z.B. nackte siRNA geschützt bis in Zytosol transportiert und freigesetzt werden. Die siRNA ist Auslöser für einen hochspezifischen RNA-Interferenz Mechanismus, der in seiner Folge Gene stillegt. Als Transporter für die siRNA eignen sich insbesondere dendritische Polyamine, da sie aufgrund ihrer multiplen Amingruppen mit dem negativ geladenen Rückgrat von Nukleinsäuren wie DNA / siRNA wechselwirken können, einen Komplex bilden, die Zellaufnahme ermöglichen, und die endosomale Freisetzung unterstützen. Auch deren strukturelle Flexibilität spielt eine entscheidende Rolle für eine effiziente Transfektion.

In Vorarbeiten wurde bereits ein dendritischen Polyglycerin (dPG) basierendes hochfunktionalisiertes Polyamin (dPG-NH_2 mit einem Aminierungsgrad von 90% und MW ~ 10 kDa) für die siRNA Transfektion *in vitro* und *in vivo* entwickelt. Allerdings ist das therapeutische Fenster dieses Vektors zu klein, sodass die *in vivo* Anwendung limitiert war. Deswegen lag der Hauptfokus dieser Doktorarbeit die multiplen strukturellen Eigenschaften des Wirkstoffträgers dPG zu variieren, um eine sicherere und effizientere siRNA Verabreichung zu gewährleisten. Zu den durchgeführten Modifikationen zählen unter anderem die Entwicklung von Polymaminen mit unterschiedlichem Funktionalisierungsgrad, die Variation der Art der eingeführten Aminfunktionalität, sowie die Größe des dPG Kerns.

Für eine Struktur-Aktivitätsstudie wurden zunächst dPG-NH_2 Analoga mit variierendem Aminierungsgrad (mit dPG MW ~10 kDa) hergestellt und mit Hinblick auf siRNA Komplexierung, Zytotoxizität, sowie Knockdown Effizienz *in vitro* und *in vivo* ausgewertet. Hierbei hat sich gezeigt, dass bestimmte Aminierungsgrade (z.B. 50% für 10 kDa dPG) für eine effiziente siRNA Transfektion notwendig sind. Bestätigt wurde dieses Ergebnis durch eine Studie an einem theoretischen Modell, welche stärkere Wechselwirkung zwischen einem 21-Basenpaar DNA Modell und einem dPG-NH_2 Analoga mit einem Aminierungsgrad von 50% und höher vorausgesagt hat.

Für den zweiten Teil dieser Arbeit wurden dPG-Amine mit hohem Molekulargewicht (dPG *Mn* ~43 kDa) und mit verschiedenen Funktionalisierungsgraden hergestellt, um das Sicherheitsprofil von dPG-NH_2 *in vivo* zu untersuchen. Die Ergebnisse der *in vivo* Studie mit dPG-NH_2 50% (dPG *Mn* ~43 kDa) zeigten eine niedrigere induzierte Immunantwort sowie eine höhere Effizienz des Luciferase Knockdowns, verglichen mit den Analoga niedrigeren Molekulargewichts (dPG MW ~10 kDa). Dabei wurde ein effektiver siRNA Vektor mit einer optimalen Ladungsdichte erhalten, dessen positive Ladung hoch genug ist, um die zelluläre Aufnahme sowie die stabile Komplexbildung mit siRNA bei niedrigen N/P Verhältnissen zu gewährleisten, jedoch niedrig genug ist, um keine Immunantwort zu induzieren.

Des weiteren wurden Arginin und Histidin an die primären Amine der äußeren Sphäre von dPG (MW ~10 kDa) gebunden, um die Zytotoxizität von dPG-NH_2 bei gleichbleibender Effektivität der siRNA Transfektion zu minimieren. Das Ergebnis war ein optimaler siRNA Vektor, der analog zum Lipofectamine® eine vergleichbare Transfektionseffizienz bei gleichzeitiger minimaler Zytotoxizität aufwies. Interessanterweise verbesserte die Einführung der Aminosäuren sowohl die *in vitro* Transfektion als auch die Zytotoxizität von dPG-NH_2.

Schließlich wurden bispezifische Antikörper mit dualer Spezifität ausgewählt, um mit dem Hapten Digoxigenin funktionalisierte Polyglycerol-basierte Prodrugs in LeY Antiköper exprimierende MCF-7 Zellen zu transportieren. In dieser Studie konnte die gezielte Bindung und Aufnahme des dPG Prodrugs nachgewiesen werden. Es konnte jedoch keine Target-spezifische Toxizität bei Digoxigenin funktionalisierten dPG Prodrugs beobachtet werden. Dies könnte an der nichtspezifischen Wechselwirkung und Aufnahme der Pro-Pharmaka liegen, die mit deren aktiver Aufnahme konkurriert.

Die in dieser Arbeit präsentierten Studien zeigen allesamt, dass das zielgerichtete und maßgeschneiderte Design der dPG-basierten Transportsysteme im Hinblick auf deren finale Anwendung von zentraler Bedeutung sind. Beispielsweise wird ein Funktionalisierungsgrad von > 50% des dPGs mit Amin benötigt um eine effiziente siRNA Transfektion *in vitro* und *in vivo* zu erreichen. Außerdem weist dPG-NH_2 mit einem Molekulargewicht von 45 kDa und einem Funktionalisierungsgrad von 50 % *in vivo* eine erhöhte siRNA Transfektion und ein besseres Toxizitätsprofil, verglichen mit seinem Analogon mit niedrigem Molekulargewicht auf. Zusammenfassend lässt sich sagen, dass sowohl die Fähigkeit der Amingruppen als Komplexbildner für die siRNA zu fungieren, als auch die Anwesenheit von Gruppen, die die endosomale Freisetzung begünstigen ausschlaggebend für eine effiziente und sichere siRNA

Transfektion sind. Als Ausblick kann die Strategie des aktiven Targetings, wie z.B. bei der Anwendung von bispezifischen Antikörpern, adaptiert werden um eine gewebespezifische siRNA Transfektion zu bewirken.

7 References

[1] R. Duncan, *Nat. Rev. Drug Discovery* **2003**, *2*, 347-360.
[2] R. Duncan, S. Dimitrijevic, E. G. Evagorou, *Stp Pharma Sciences* **1996**, *6*, 237-263.
[3] R. Duncan, M. J. Vicent, *Adv. Drug Delivery Rev.* **2013**, *65*, 60-70.
[4] R. Duncan, R. Gaspar, *Mol. Pharmaceutics* **2011**, *8*, 2101-2141.
[5] H. A. Shelanski, M. V. Shelanski, *J. Int. Coll. Surg.* **1956**, *25*, 727-734.
[6] A. V. Kabanov, E. V. Batrakova, V. Y. Alakhov, *Adv. Drug Delivery Rev.* **2002**, *54*, 759-779.
[7] T. Nakanishi, S. Fukushima, K. Okamoto, M. Suzuki, Y. Matsumura, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, *J. Controlled Release* **2001**, *74*, 295-302.
[8] Y. Matsumura, K. Kataoka, *Cancer Sci.* **2009**, *100*, 572-579.
[9] R. Duncan, *Nat. Revi. Cancer* **2006**, *6*, 688-701.
[10] R. Soleyman, M. Adeli, *Polym. Chem.* **2015**, *6*, 10-24.
[11] L. Y. T. Chou, K. Ming, W. C. W. Chan, *Chem. Soc. Rev.* **2011**, *40*, 233-245.
[12] R. Duncan, S. C. W. Richardson, *Molecul. Pharmaceutics* **2012**, *9*, 2380-2402.
[13] M. Yanagishita, V. C. Hascall, *J. Biol. Chem.* **1992**, *267*, 9451-9454.
[14] S. D. Conner, S. L. Schmid, *Nature* **2003**, *422*, 37-44.
[15] A. I. Tauber, *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 897-901.
[16] A. Aderem, D. M. Underhill, *Annu. Rev. Immunol.* **1999**, *17*, 593-623.
[17] S. L. Schmid, *Annu. Rev. Biochem.* **1997**, *66*, 511-548.
[18] L. Pelkmans, A. Helenius, *Traffic* **2002**, *3*, 311-320.
[19] H. Damke, T. Baba, A. M. Vanderbliek, S. L. Schmid, *J. Cell Biol.* **1995**, *131*, 69-80.
[20] H. T. McMahon, E. Boucrot, *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 517-533.
[21] N. Larson, H. Ghandehari, *Chem. of Mater.* **2012**, *24*, 840-853.
[22] Y. Matsumura, H. Maeda, *Cancer Res.* **1986**, *46*, 6387-6392.
[23] R. K. Jain, *Adv. Drug Delivery Rev.* **2001**, *46*, 149-168.
[24] J. Wu, T. Akaike, H. Maeda, *Cancer Res.* **1998**, *58*, 159-165.
[25] A. A. Gabizon, H. Shmeeda, S. Zalipsky, *J. Liposome Res.* **2006**, *16*, 175-183.
[26] L. W. Seymour, Y. Miyamoto, H. Maeda, M. Brereton, J. Strohalm, K. Ulbrich, R. Duncan, *Eur. J. Cancer* **1995**, *31A*, 766-770.
[27] J. W. Singer, B. Baker, P. De Vries, A. Kumar, S. Shaffer, E. Vawter, M. Bolton, P. Garzone, in *Polymer Drugs in the Clinical Stage: Advantages and Prospects, Vol. 519* (Eds.: H. Maeda, A. Kabanov, K. Kataoka, T. Okano), **2003**, pp. 81-99.
[28] I. Brigger, C. Dubernet, P. Couvreur, *Adv. Drug Delivery Rev.* **2002**, *54*, 631-651.
[29] Q. Que, R. A. Jorgensen, *Developmental Genetics* **1998**, *22*, 100-109.
[30] A. Fire, S. Q. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello, *Nature* **1998**, *391*, 806-811.
[31] A. P. McCaffrey, L. Meuse, T.-T. T. Pham, D. S. Conklin, G. J. Hannon, M. A. Kay, *Nature* **2002**, *418*, 38-39.
[32] M. E. Davis, J. E. Zuckerman, C. H. J. Choi, D. Seligson, A. Tolcher, C. A. Alabi, Y. Yen, J. D. Heidel, A. Ribas, *Nature* **2010**, *464*, 1067-U1140.
[33] S. Guo, K. J. Kemphues, *Cell* **1995**, *81*, 611-620.
[34] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature* **2001**, *411*, 494-498.
[35] A. Grunweller, R. K. Hartmann, *Curr. Med. Chem.* **2005**, *12*, 3143-3161.
[36] I. Bantounas, L. A. Phylactou, J. B. Uney, *J. Mol. Endocrinol.* **2004**, *33*, 545-557.
[37] D. M. Dykxhoorn, C. D. Novina, P. A. Sharp, *Nat Rev Mol Cell Biol* **2003**, *4*, 457-467.
[38] S. W. Jones, P. M. d. Souza, M. A. Lindsay, *Curr. Opin. Pharmacol.* **2004**, *4*, 522-527.
[39] J. M. Perkel, *Science* **2009**, *326*, 454-456.

[40] L. He, G. J. Hannon, *Nat Rev Genet* **2004**, *5*, 522-531.
[41] G. Meister, T. Tuschl, *Nature* **2004**, *431*, 343-349.
[42] Y. W. Kong, D. Ferland-McCollough, T. J. Jackson, M. Bushell, *The Lancet Oncology* **2012**, *13*, e249-e258.
[43] E. Wagner, *Angew. Chem., Int. Ed.* **2015**, *54*, 5824-5826.
[44] R. L. Kanasty, K. A. Whitehead, A. J. Vegas, D. G. Anderson, *Mol Ther* **2012**, *20*, 513-524.
[45] K. A. Whitehead, R. Langer, D. G. Anderson, *Nat Rev Drug Discov* **2009**, *8*, 129-138.
[46] A. L. Jackson, P. S. Linsley, *Nat. Rev. Drug Discovery* **2010**, *9*, 57-67.
[47] D. N. Nguyen, K. P. Mahon, G. Chikh, P. Kim, H. Chung, A. P. Vicari, K. T. Love, M. Goldberg, S. Chen, A. M. Krieg, J. Chen, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, E797-E803.
[48] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres, G. Hartmann, *Nat. Med.* **2005**, *11*, 263-270.
[49] A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock, I. MacLachlan, *Nat. Biotechnol.* **2005**, *23*, 457-462.
[50] A. D. Judge, G. Bola, A. C. H. Lee, I. MacLachlan, *Mol Ther* **2006**, *13*, 494-505.
[51] K. A. Whitehead, R. Langer, D. G. Anderson, *Nat Rev Drug Discov* **2010**, *9*, 412-412.
[52] A. Gallas, C. Alexander, M. C. Davies, S. Puri, S. Allen, *Chem. Soc. Rev.* **2013**, *42*, 7983-7997.
[53] C. Wolfrum, S. Shi, K. N. Jayaprakash, M. Jayaraman, G. Wang, R. K. Pandey, K. G. Rajeev, T. Nakayama, K. Charrise, E. M. Ndungo, T. Zimmermann, V. Koteliansky, M. Manoharan, M. Stoffel, *Nat. Biotechnol.* **2007**, *25*, 1149-1157.
[54] K. Nishina, T. Unno, Y. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa, T. Yokota, *Mol. Ther.* **2008**, *16*, 734-740.
[55] M. E. Kleinman, K. Yamada, A. Takeda, V. Chandrasekaran, M. Nozaki, J. Z. Baffi, R. J. C. Albuquerque, S. Yamasaki, M. Itaya, Y. Pan, B. Appukuttan, D. Gibbs, Z. Yang, K. Kariko, B. K. Ambati, T. A. Wilgus, L. A. DiPietro, E. Sakurai, K. Zhang, J. R. Smith, E. W. Taylor, J. Ambati, *Nature* **2008**, *452*, 591-597.
[56] T. Inoue, M. Sugimoto, T. Sakurai, R. Saito, N. Futaki, Y. Hashimoto, Y. Honma, I. Arai, S. Nakaike, *J. Gene Med.* **2007**, *9*, 994-1001.
[57] D. Palliser, D. Chowdhury, Q. Y. Wang, S. J. Lee, R. T. Bronson, D. M. Knipe, J. Lieberman, *Nature* **2006**, *439*, 89-94.
[58] B. J. Li, Q. Q. Tang, D. Cheng, C. Qin, F. Y. Xie, Q. Wei, J. Xu, Y. J. Liu, B. J. Zheng, M. C. Woodle, N. S. Zhong, P. Y. Lu, *Nat. Med.* **2005**, *11*, 944-951.
[59] M. DiFiglia, M. Sena-Esteves, K. Chase, E. Sapp, E. Pfister, M. Sass, J. Yoder, P. Reeves, R. K. Pandey, K. G. Rajeev, M. Manoharan, D. W. Y. Sah, P. D. Zamore, N. Aronin, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 17204-17209.
[60] P. H. Tan, L. C. Yang, H. C. Shih, K. C. Lan, J. T. Cheng, *Gene Ther.* **2005**, *12*, 59-66.
[61] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H. P. Vornlocher, *Nature* **2004**, *432*, 173-178.
[62] Y. Omidi, A. J. Hollins, R. M. Drayton, S. Akhtar, *J Drug Target* **2005**, *13*, 431-443.
[63] O. Ishida, K. Maruyama, K. Sasaki, M. Iwatsuru, *Int J Pharm* **1999**, *190*, 49-56.
[64] H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin, D. G. Anderson, *Nat Rev Genet* **2014**, *15*, 541-555.

[65] K. A. Whitehead, R. Langer, D. G. Anderson, *Nat. Rev. Drug Discov.* **2009**, *8*, 129-138.
[66] P. D. Richardson, L. B. Augustin, B. T. Kren, C. J. Steer, *Stem Cells* **2002**, *20*, 105-118.
[67] I. M. Verma, N. Somia, *Nature* **1997**, *389*, 239-242.
[68] H. Shen, T. Sun, M. Ferrari, *Cancer Gene Ther.* **2012**, *19*, 367-373.
[69] M. Giacca, S. Zacchigna, *J. Controlled Release* **2012**, *161*, 377-388.
[70] Y. Lee, K. Kataoka, *Nucleic Acid Drugs* **2012**, *249*, 95-134.
[71] M. V. Chengalvala, M. D. Lubeck, B. J. Selling, R. J. Natuk, K. H. Hsu, B. B. Mason, P. K. Chanda, R. A. Bhat, B. M. Bhat, S. Mizutani, *Curr. Opin. Biotechnol.* **1991**, *2*, 718-722.
[72] B. J. Carter, *Curr. Opin. Biotechnol.* **1992**, *3*, 533-539.
[73] J. R. Morgan, R. G. Tompkins, M. L. Yarmush, *Adv. Drug Delivery Rev.* **1993**, *12*, 143-158.
[74] E. Marshall, *Science* **1999**, *286*, 2244-2245.
[75] J. Kaiser, *Science* **2003**, *299*, 495-495.
[76] M. Ashtari, L. L. Cyckowski, J. F. Monroe, K. A. Marshall, D. C. Chung, A. Auricchio, F. Simonelli, B. P. Leroy, A. M. Maguire, K. S. Shindler, J. Bennett, *J. Clin. Invest.* **2011**, *121*, 2160-2168.
[77] M. Cavazzana-Calvo, A. Fischer, *J. Clin. Invest.* **2007**, *117*, 1456-1465.
[78] S. Li, L. Huang, *Gene Ther.* **2000**, *7*, 31-34.
[79] S. Chesnoy, L. Huang, *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 27-47.
[80] E. Wagner, *Mol. Ther.* **2008**, *16*, 1-2.
[81] E. Wagner, *Acc. Chem. Res.* **2012**, *45*, 1005-1013.
[82] P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.* **1987**, *84*, 7413-7417.
[83] F. Liu, Y. K. Song, D. Liu, *Gene Ther.* **1999**, *6*, 1258-1266.
[84] S. L. Li, *Curr. Gene Ther.* **2004**, *4*, 309-316.
[85] D. L. Miller, S. V. Pislaru, J. E. Greenleaf, *Somatic Cell Mol. Genet.* **2002**, *27*, 115-134.
[86] Y. Zhang, L. C. Yu, *Curr. Opin. Biotechnol.* **2008**, *19*, 506-510.
[87] A. Sharei, J. Zoldan, A. Adamo, W. Y. Sim, N. Cho, E. Jackson, S. Mao, S. Schneider, M.-J. Han, A. Lytton-Jean, P. A. Basto, S. Jhunjhunwala, J. Lee, D. A. Heller, J. W. Kang, G. C. Hartoularos, K.-S. Kim, D. G. Anderson, R. Langer, K. F. Jensen, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 2082-2087.
[88] M. A. Mintzer, E. E. Simanek, *Chem. Rev.* **2009**, *109*, 259-302.
[89] A. Schroeder, C. G. Levins, C. Cortez, R. Langer, D. G. Anderson, *J. Intern. Med.* **2010**, *267*, 9-21.
[90] S. C. Semple, A. Akinc, J. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Y. Sah, D. Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden, M. J. Hope, *Nat. Biotechnol.* **2010**, *28*, 172-U118.
[91] J. Hoyer, I. Neundorf, *Acc. of Chem. Res.* **2012**, *45*, 1048-1056.
[92] K. Raemdonck, T. F. Martens, K. Braeckmans, J. Demeester, S. C. De Smedt, *Adv. Drug Delivery Rev.* **2013**, *65*, 1123-1147.

[93] K. A. Howard, U. L. Rahbek, X. D. Liu, C. K. Damgaard, S. Z. Glud, M. O. Andersen, M. B. Hovgaard, A. Schmitz, J. R. Nyengaard, F. Besenbacher, J. Kjems, *Mol. Ther.* **2006**, *14*, 476-484.
[94] Z. Liu, Y. Jiao, Y. Wang, C. Zhou, Z. Zhang, *Adv. Drug Delivery Rev.* **2008**, *60*, 1650-1662.
[95] S. Mao, W. Sun, T. Kissel, *Adv. Drug Delivery Rev.* **2010**, *62*, 12-27.
[96] S. B. Rao, C. P. Sharma, *J. Biomed. Mater. Res.* **1997**, *34*, 21-28.
[97] C. P. Gomes, C. D. F. Lopes, P. M. D. Moreno, A. Varela-Moreira, M. J. Alonso, A. P. Pego, *MRS Bull.* **2014**, *39*, 60-70.
[98] M. Huang, C. W. Fong, E. Khor, L. Y. Lim, *J. of Controlled Release* **2005**, *106*, 391-406.
[99] T. Kiang, H. Wen, H. W. Lim, K. W. Leong, *Biomaterials* **2004**, *25*, 5293-5301.
[100] K. Wong, G. B. Sun, X. Q. Zhang, H. Dai, Y. Liu, C. B. He, K. W. Leong, *Bioconjugate Chem* **2006**, *17*, 152-158.
[101] J. Malmo, H. Sorgard, K. M. Varum, S. P. Strand, *J. Controlled Release* **2012**, *158*, 261-268.
[102] H. Gonzalez, S. J. Hwang, M. E. Davis, *Bioconjugate Chem.* **1999**, *10*, 1068-1074.
[103] M. E. Davis, M. E. Brewster, *Nat. Rev. Drug Discovery* **2004**, *3*, 1023-1035.
[104] S. Mishra, J. D. Heidel, P. Webster, M. E. Davis, *J. Controlled Release* **2006**, *116*, 179-191.
[105] R. Kanasty, J. R. Dorkin, A. Vegas, D. Anderson, *Nat. Mater.* **2013**, *12*, 967-977.
[106] S. H. Pun, M. E. Davis, *Bioconjugate Chem.* **2002**, *13*, 630-639.
[107] S. Mishra, P. Webster, M. E. Davis, *Eur. J. of Cell Biol.* **2004**, *83*, 97-111.
[108] D. W. Bartlett, H. Su, I. J. Hildebrandt, W. A. Weber, M. E. Davis, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 15549-15554.
[109] S. Hu-Lieskovan, J. D. Heidel, D. W. Bartlett, M. E. Davis, T. J. Triche, *Cancer Res.* **2005**, *65*, 8984-8992.
[110] J. D. Heidel, Z. Yu, J. Y.-C. Liu, S. M. Rele, Y. Liang, R. K. Zeidan, D. J. Kornbrust, M. E. Davis, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 5715-5721.
[111] R. Duncan, *Nat. Rev. Cancer* **2006**, *6*, 688-701.
[112] M. Martinelli, M. Calderon, C. I. Alvarez, M. C. Strumia, *React. Funct. Polym.* **2007**, *67*, 1018-1026.
[113] B. Devarakonda, D. P. Otto, A. A. Judefeind, R. A. Hill, M. M. de Villiers, *Int. J. Pharm.* **2007**, *345*, 142-153.
[114] H. Tuerk, A. Shukla, P. C. A. Rodrigues, H. Rehage, R. Haag, *Chem. - Eur. J.* **2007**, *13*, 4187-4196.
[115] J. L. Mynar, T. J. Lowery, D. E. Wemmer, A. Pines, J. M. J. Frechet, *J. Am. Chem. Soc.* **2006**, *128*, 6334-6335.
[116] V. S. Talanov, C. A. S. Regino, H. Kobayashi, M. Bernardo, P. L. Choyke, M. W. Brechbiel, *Nano Letters* **2006**, *6*, 1459-1463.
[117] H. Kobayashi, M. W. Brechbiel, *Adv. Drug Delivery Rev.* **2005**, *57*, 2271-2286.
[118] S. Hecht, J. M. J. Frechet, *Angew. Chem., Int. Ed. Engl.* **2001**, *40*, 74-91.
[119] N. N. Ma, C. Ma, Y. Deng, T. Wang, N. Y. He, *J. Nanosci. Nanotechnol.* **2013**, *13*, 33-39.
[120] L. A. Tziveleka, A. M. G. Psarra, D. Tsiourvas, C. M. Paleos, *Int. J. Pharm.* **2008**, *356*, 314-324.
[121] P. Kesharwani, K. Jain, N. K. Jain, *Prog. Polym. Sci.* **2014**, *39*, 268-307.
[122] S. Battah, S. Balaratnam, A. Casas, S. O'Neill, C. Edwards, A. Batlle, P. Dobbin, A. J. MacRobert, *Mol. Cancer Ther.* **2007**, *6*, 876-885.

[123] G. D. Zhang, A. Harada, N. Nishiyama, D. L. Jiang, H. Koyama, T. Aida, K. Kataoka, *J. of Controlled Release* **2003**, *93*, 141-150.
[124] S. E. Stiriba, H. Frey, R. Haag, *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 1329-1334.
[125] R. Guo, X. Y. Shi, *Curr. Drug Metab.* **2012**, *13*, 1097-1109.
[126] D. A. Tomalia, J. M. J. Frechet, *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 2719-2728.
[127] C. R. Dick, G. E. Ham, *J. Macromol. Sci., Chem.* **1970**, *A 4*, 1301-&.
[128] O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 7297-7301.
[129] D. Fischer, T. Bieber, Y. X. Li, H. P. Elsasser, T. Kissel, *Pharm. Res.* **1999**, *16*, 1273-1279.
[130] K. Kunath, A. von Harpe, D. Fischer, H. Peterson, U. Bickel, K. Voigt, T. Kissel, *J. of Controlled Release* **2003**, *89*, 113-125.
[131] H. Petersen, P. M. Fechner, A. L. Martin, K. Kunath, S. Stolnik, C. J. Roberts, D. Fischer, M. C. Davies, T. Kissel, *Bioconjugate Chem.* **2002**, *13*, 845-854.
[132] S. Mao, M. Neu, O. Germershaus, O. Merkel, J. Sitterberg, U. Bakowsky, T. Kissel, *Bioconjugate Chem.* **2006**, *17*, 1209-1218.
[133] B. Liang, M. L. He, C. Y. Chan, Y. C. Chen, X. P. Li, Y. Li, D. X. Zheng, M. C. Lin, H. F. Kung, X. T. Shuai, Y. Peng, *Biomaterials* **2009**, *30*, 4014-4020.
[134] S. Boeckle, J. Fahrmeir, W. Roedl, M. Ogris, E. Wagner, *J. Controlled Release* **2006**, *112*, 240-248.
[135] M. Meyer, A. Philipp, R. Oskuee, C. Schmidt, E. Wagner, *J. Am. Chem. Soc.* **2008**, *130*, 3272-+.
[136] N. Tietze, J. Pelisek, A. Philipp, W. Roedl, T. Merdan, P. Tarcha, M. Ogris, E. Wagner, *Oligonucleotides* **2008**, *18*, 161-174.
[137] D. Schaffert, C. Troiber, E. E. Salcher, T. Froehlich, I. Martin, N. Badgujar, C. Dohmen, D. Edinger, R. Klaeger, G. Maiwald, K. Farkasova, S. Seeber, K. Jahn-Hofmann, P. Hadwiger, E. Wagner, *Angew. Chem., Int. Ed.* **2011**, *50*, 8986-8989.
[138] D. A. Tomalia, H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder, P. Smith, *Polymer Journal* **1985**, *17*, 117-132.
[139] J. Haensler, F. C. Szoka, *Bioconjugate Chem.* **1993**, *4*, 372-379.
[140] J. F. KukowskaLatallo, A. U. Bielinska, J. Johnson, R. Spindler, D. A. Tomalia, J. R. Baker, *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 4897-4902.
[141] A. U. Bielinska, J. F. KukowskaLatallo, J. R. Baker, *Biochim. Biophys. Acta, Gene Struct. Expression* **1997**, *1353*, 180-190.
[142] N. D. Sonawane, F. C. Szoka, A. S. Verkman, *J. Biol. Chem.* **2003**, *278*, 44826-44831.
[143] J. H. Lee, Y. B. Lim, J. S. Choi, Y. Lee, T. I. Kim, H. J. Kim, J. K. Yoon, K. Kim, J. S. Park, *Bioconjugate Chem.* **2003**, *14*, 1214-1221.
[144] M. X. Tang, C. T. Redemann, F. C. Szoka, *Bioconjugate Chem.* **1996**, *7*, 703-714.
[145] M. Kramer, J. F. Stumbe, G. Grimm, B. Kaufmann, U. Kruger, M. Weber, R. Haag, *Chembiochem* **2004**, *5*, 1081-1087.
[146] J. Y. Wu, J. H. Zhou, F. Q. Qu, P. H. Bao, Y. Zhang, L. Peng, *Chem. Commun.* **2005**, 313-315.
[147] X.-C. Shen, J. Zhou, X. Liu, J. Wu, F. Qu, Z.-L. Zhang, D.-W. Pang, G. Quelever, C.-C. Zhang, L. Peng, *Organic & Biomolecular Chemistry* **2007**, *5*, 3674-3681.
[148] X.-x. Liu, P. Rocchi, F.-q. Qu, S.-q. Zheng, Z.-c. Liang, M. Gleave, J. Iovanna, L. Peng, *Chemmedchem* **2009**, *4*, 1302-1310.
[149] J. Zhou, C. P. Neff, X. Liu, J. Zhang, H. Li, D. D. Smith, P. Swiderski, T. Aboellail, Y. Huang, Q. Du, Z. Liang, L. Peng, R. Akkina, J. J. Rossi, *Mol. Ther.* **2011**, *19*, 2228-2238.

[150] X. Liu, J. Wu, M. Yammine, J. Zhou, P. Posocco, S. Viel, C. Liu, F. Ziarelli, M. Fermeglia, S. Pricl, G. Victorero, N. Catherine, P. Erbacher, J.-P. Behr, L. Peng, *Bioconjugate Chem.* **2011**, *22*, 2461-2473.
[151] J. S. Choi, K. Nam, J. Park, J. B. Kim, J. K. Lee, J. Park, *J. Controlled Release* **2004**, *99*, 445-456.
[152] T.-i. Kim, J.-u. Baek, J. K. Yoon, J. S. Choi, K. Kim, J.-s. Park, *Bioconjugate Chem.* **2007**, *18*, 309-317.
[153] Q. Yuan, E. Lee, W. A. Yeudall, H. Yang, *Oral Oncol.* **2010**, *46*, 698-704.
[154] M. Calderon, M. A. Quadir, S. K. Sharma, R. Haag, *Advanced Materials* **2010**, *22*, 190-218.
[155] Q. Wei, S. Krysiak, K. Achazi, T. Becherer, P.-L. M. Noeske, F. Paulus, H. Liebe, I. Grunwald, J. Dernedde, A. Hartwig, T. Hugel, R. Haag, *Colloids and Surfaces B-Biointerfaces* **2014**, *122*, 684-692.
[156] T. Rossow, J. A. Heyman, A. J. Ehrlicher, A. Langhoff, D. A. Weitz, R. Haag, S. Seiffert, *J. Am. Chem. Soc.* **2012**, *134*, 4983-4989.
[157] A. F. Hussain, H. R. Krueger, F. Kampmeier, T. Weissbach, K. Licha, F. Kratz, R. Haag, M. Calderon, S. Barth, *Biomacromolecules* **2013**, *14*, 2510-2520.
[158] M. Calderon, P. Welker, K. Licha, I. Fichtner, R. Graeser, R. Haag, F. Kratz, *J. Controlled Release* **2011**, *151*, 295-301.
[159] R. Haag, F. Kratz, *Angew. Chem., Int. Ed.* **2006**, *45*, 1198-1215.
[160] S. Roller, H. Zhou, R. Haag, Molecular Diversity 2005, 9, 305-316.
[161] W. Fischer, M. Calderón, A. Schulz, I. Andreou, W. Weber, R. Haag, *Bioconjugate Chem.* **2010**, *21*, 1744-1852.
[162] P. Ofek, W. Fischer, M. Calderón, R. Haag, R. Satchi-Fainaro, *The FASEB Journal* **2010**, *24*, 3122-3134.
[163] W. Fischer, M. A. Quadir, A. Barnard, D. K. Smith, R. Haag, *Macromol. Biosci.* **2011**, *11*, 1736-1746.
[164] S. Malhotra, H. Bauer, A. Tschiche, A. M. Staedtler, A. Mohr, M. Calderon, V. S. Parmar, L. Hoeke, S. Sharbati, R. Einspanier, R. Haag, *Biomacromolecules* **2012**, *13*, 3087-3098.
[165] R. J. Christie, Y. Matsumoto, K. Miyata, T. Nomoto, S. Fukushima, K. Osada, J. Halnaut, F. Pittella, H. J. Kim, N. Nishiyama, K. Kataoka, *Acs Nano* **2012**, *6*, 5174-5189.
[166] H. Yu, Y. Nie, C. Dohmen, Y. Li, E. Wagner, *Biomacromolecules* **2011**, *12*, 2039-2047.
[167] R. Xu, X.-L. Wang, Z.-R. Lu, *Langmuir* **2010**, *26*, 13874-13882.
[168] A. Barnard, P. Posocco, S. Pricl, M. Calderon, R. Haag, M. E. Hwang, V. W. T. Shum, D. W. Pack, D. K. Smith, *J. Am. Chem. Soc.* **2011**, *133*, 20288-20300.
[169] Z. X. Li, J. C. Barnes, A. Bosoy, J. F. Stoddart, J. I. Zink, *Chem. Soc. Rev.* **2012**, *41*, 2590-2605.
[170] T. Yu, X. Liu, A.-L. Bolcato-Bellemin, Y. Wang, C. Liu, P. Erbacher, F. Qu, P. Rocchi, J.-P. Behr, L. Peng, *Angew. Chem., Int. Ed. Engl.* **2012**, *51*, 8478-8484.
[171] S. Malhotra, M. Calderon, A. K. Prasad, V. S. Parmar, R. Haag, *Org. Biomol. Chem.* **2010**, *8*, 2228-2237.
[172] R. Fraley, S. Subramani, P. Berg, D. Papahadjopoulos, *J. Biol. Chem.* **1980**, *255*, 431-435.
[173] R. W. Malone, P. L. Felgner, I. M. Verma, *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86*, 6077-6081.
[174] T. S. Zimmermann, A. C. H. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M. N. Fedoruk, J. Harborth, J. A. Heyes, L. B. Jeffs, M. John, A. D. Judge, K. Lam, K. McClintock, L.

V. Nechev, L. R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A. J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H. P. Vornlocher, I. MacLachlan, *Nature* **2006**, *441*, 111-114.

[175] M. A. Quadir, *Dendritic polymers as nanoscale delivery systems for metal ion and drugs (PhD thesis) FU Berlin, Germany.*

[176] D. Zhi, S. Zhang, B. Wang, Y. Zhao, B. Yang, S. Yu, *Bioconjugate Chem.* **2010**, *21*, 563-577.

[177] J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, P. L. Felgner, *J. Biol. Chem.* **1994**, *269*, 2550-2561.

[178] J. A. Heyes, D. Niculescu-Duvaz, R. G. Cooper, C. J. Springer, *J. Med. Chem.* **2002**, *45*, 99-114.

[179] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Scherman, *J. Med. Chem.* **1998**, *41*, 224-235.

[180] A. Unciti-Broceta, M. N. Bacon, M. Bradley, *Nucleic Acid Transfection* **2010**, *296*, 15-49.

[181] P. Hahn, E. Scanlan, in *Nucleic Acid Transfection in Topics in Current Chemistry, Vol. 296* (Eds.: W. Bielke, C. Erbacher), Springer Berlin/ Heidelberg, **2010**, pp. 1-13.

[182] M. Elfinger, S. Uezguen, C. Rudolph, *Curr. Nanosci.* **2008**, *4*, 322-353.

[183] W. Li, F. C. Szoka, Jr., *Pharm. Res.* **2007**, *24*, 438-449.

[184] B. Martin, M. Sainlos, A. Aissaoui, N. Oudrhiri, M. Hauchecorne, J. P. Vigneron, J. M. Lehn, P. Lehn, *Curr. Pharm. Des.* **2005**, *11*, 375-394.

[185] A. R. Gascon, J. Luis Pedraz, *Expert Opin. Ther. Pat.* **2008**, *18*, 515-524.

[186] J. M. Boon, B. D. Smith, *Curr. Opin. Chem. Biol.* **2002**, *6*, 749-756.

[187] V. P. Torchilin, *Eur. J. Pharm. Sci.* **2000**, *11*, S81-S91.

[188] E. S. Vitetta, K. A. Krolick, M. Miyamainaba, W. Cushley, J. W. Uhr, *Science* **1983**, *219*, 644-650.

[189] H. Ringsdorf, *J. Polym. Sci., Part C: Polym. Symp.* **1975**, 135-153.

[190] D. Bhadra, S. Bhadra, S. Jain, N. K. Jain, *Int. J. Pharm.* **2003**, *257*, 111-124.

[191] D. Peer, J. M. Karp, S. Hong, O. C. FaroKhzad, R. Margalit, R. Langer, *Nat. Nanotechnol.* **2007**, *2*, 751-760.

[192] F. Kratz, I. A. Muller, C. Ryppa, A. Warnecke, *Chemmedchem* **2008**, *3*, 20-53.

[193] E. Vives, J. Schmidt, A. Pelegrin, *Biochim. Biophys. Acta, Rev. Cancer* **2008**, *1786*, 126-138.

[194] M. Di Benedetto, A. Starzec, R. Vassy, G.-Y. Perret, M. Crepin, *Biochim. Biophys. Acta, Gen. Subj.* **2008**, *1780*, 723-732.

[195] J. F. Ren, S. Shen, D. G. Wang, Z. J. Xi, L. R. Guo, Z. Q. Pang, Y. Qian, X. Y. Sun, X. G. Jiang, *Biomaterials* **2012**, *33*, 3324-3333.

[196] T. R. Daniels, T. Delgado, G. Helguera, M. L. Penichet, *Clinical Immunology* **2006**, *121*, 159-176.

[197] C. Leuschner, C. S. S. R. Kumar, W. Hansel, W. Soboyejo, J. Zhou, J. Hormes, *Breast Cancer Res. Treat.* **2006**, *99*, 163-176.

[198] X. Zhao, H. Li, R. J. Lee, *Expert Opin. Drug Delivery* **2008**, *5*, 309-319.

[199] P. S. Low, W. A. Henne, D. D. Doorneweerd, *Acc. Chem. Res.* **2008**, *41*, 120-129.

[200] J. Beuttler, M. Rothdiener, D. Mueller, F. Y. Frejd, R. E. Kontermann, *Bioconjugate Chem.* **2009**, *20*, 1201-1208.

[201] E. Kopansky, Y. Shamay, A. David, *J. Drug Targeting* **2011**, *19*, 933-943.

[202] M. J. Mitchell, C. S. Chen, V. Ponmudi, A. D. Hughes, M. R. King, *J. Controlled Release* **2012**, *160*, 609-617.

[203] Y. Zhang, S. G. Xing, Z. Wang, Q. H. Kang, Y. Ling, M. Y. Yao, Y. P. He, Y. Jin, X. G. Chu, *Prog. Biochem. Biophys.* **2015**, *42*, 236-243.

[204] X. Li, Q. Zhao, L. Qiu, *J. Controlled Release* **2013**, *171*, 152-162.
[205] J. Folkman, M. Bach, J. W. Rowe, F. Davidoff, P. Lambert, C. Hirsch, A. Goldberg, H. H. Hiatt, J. Glass, E. Henshaw, *N. Engl. J. Med.* **1971**, *285*, 1182-&.
[206] C. Francavilla, L. Maddaluno, U. Cavallaro, *Semin. Cancer Biol.* **2009**, *19*, 298-309.
[207] F. G. Giancotti, E. Ruoslahti, *Science* **1999**, *285*, 1028-1032.
[208] K. Switala-Jelen, K. Dabrowska, A. Opolski, L. Lipinska, M. Nowaczyk, A. Gorski, *Folia Biologica* **2004**, *50*, 143-152.
[209] C. J. Avraamides, B. Garmy-Susini, J. A. Varner, *Nat. Rev. Cancer* **2008**, *8*, 604-617.
[210] S. Zitzmann, V. Ehemann, M. Schwab, *Cancer Res.* **2002**, *62*, 5139-5143.
[211] E. Ruoslahti, M. D. Pierschbacher, *Science* **1987**, *238*, 491-497.
[212] S. Liu, *Bioconjugate chem.* **2009**, *20*, 2199-2213.
[213] S. Verrier, S. Pallu, R. Bareille, A. Jonczyk, J. Meyer, M. Dard, J. Amedee, *Biomaterials* **2002**, *23*, 585-596.
[214] F. Wang, Y. Li, Y. Shen, A. Wang, S. Wang, T. Xie, *Int. J. Mol. Sci.* **2013**, *14*, 13447.
[215] F. Danhier, A. Le Breton, V. Preat, *Mol. Pharm.* **2012**, *9*, 2961-2973.
[216] S. Meng, B. Su, W. Li, Y. Ding, L. Tang, W. Zhou, Y. Song, Z. Caicun, *Medical Oncology* **2011**, *28*, 1180-1187.
[217] C. Zhan, B. Gu, C. Xie, J. Li, Y. Liu, W. Lu, *J. Controlled Release* **2010**, *143*, 136-142.
[218] F. Danhier, V. Pourcelle, J. Marchand-Brynaert, C. Jerome, O. Feron, V. Preat, in *Nanomedicine: Cancer, Diabetes, and Cardiovascular, Central Nervous System, Pulmonary and Inflammatory Diseases, Vol. 508* (Ed.: N. Duzgunes), **2012**, pp. 157-175.
[219] R. M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Q. Tang, G. Storm, G. Molema, P. Y. Lu, P. V. Scaria, M. C. Woodle, *Nucleic Acids Res.* **2004**, *32*.
[220] H. D. Han, L. S. Mangala, J. W. Lee, M. M. K. Shahzad, H. S. Kim, D. Shen, E. J. Nam, E. M. Mora, R. L. Stone, C. Lu, S. J. Lee, J. W. Roh, A. M. Nick, G. Lopez-Berestein, A. K. Sood, *Clin. Cancer Res.* **2010**, *16*, 3910-3922.
[221] K. Numata, J. Hamasaki, B. Subramanian, D. L. Kaplan, *J. Controlled Release* **2010**, *146*, 136-143.
[222] J. A. Jiang, S. J. Yang, J. C. Wang, L. J. Yang, Z. Z. Xu, T. Yang, X. Y. Liu, Q. A. Zhang, *Eur. J. Pharm. Biopharm.* **2010**, *76*, 170-178.
[223] T. L. Kaneshiro, Z. R. Lu, *Biomaterials* **2009**, *30*, 5660-5666.
[224] H. W. Schroeder, L. Cavacini, *J. Allergy Clin. Immunol.* **2010**, *125*, S41-S52.
[225] K. A. Smith, P. N. Nelson, P. Warren, S. J. Astley, P. G. Murray, J. Greenman, *J. Clin. Pathol.* **2004**, *57*, 912-917.
[226] H. M. Warenius, G. Galfre, N. M. Bleehen, C. Milstein, *Eur. J. Cancer Clin. Oncol.* **1981**, *17*, 1009-1015.
[227] J. E. Bakema, M. van Egmond, in *Fc Receptors, Vol. 382* (Eds.: M. Daeron, F. Nimmerjahn), **2014**, pp. 373-392.
[228] J. S. James, G. Dubs, *AIDS treatment news* **1997**, 2-3.
[229] J. Albanell, J. Baselga, *Drugs Today* **1999**, *35*, 931-946.
[230] N. Ferrara, *Oncology* **2005**, *69*, 11-16.
[231] P. Trail, *Antibodies* **2013**, *2*, 113-129.
[232] S. Panowski, S. Bhakta, H. Raab, P. Polakis, J. R. Junutula, *Mabs* **2014**, *6*, 34-45.
[233] D. J. Elias, L. Hirschowitz, L. E. Kline, J. F. Kroener, R. O. Dillman, L. E. Walker, J. A. Robb, R. M. Timms, *Cancer Res.* **1990**, *50*, 4154-4159.
[234] A. W. Tolcher, S. Sugarman, K. A. Gelmon, R. Cohen, M. Saleh, C. Isaacs, L. Young, D. Healey, N. Onetto, W. Slichenmyer, *J. Clin. Oncol.* **1999**, *17*, 478-484.
[235] T. M. Allen, *Nat. Rev. Cancer* **2002**, *2*, 750-763.
[236] M. Steiner, D. Neri, *Clin. Cancer Res.* **2011**, *17*, 6406-6416.

[237] R. E. Kontermann, U. Brinkmann, *Drug Discovery Today* **2015**, *in press*.
[238] D. Mueller, R. E. Kontermann, *Biodrugs* **2010**, *24*, 89-98.
[239] U. H. Weidle, G. Tiefenthaler, E. H. Weiss, G. Georges, U. Brinkmann, *Cancer Genomics Proteomics* **2013**, *10*, 1-18.
[240] D. Müller, R. Kontermann, *BioDrugs* **2010**, *24*, 89-98.
[241] M. M. Heiss, M. A. Strohlein, M. Jager, R. Kimmig, A. Burges, A. Schoberth, K. W. Jauch, F. W. Schildberg, H. Lindhofer, *Int. J. Cancer* **2005**, *117*, 435-443.
[242] B. Schneider, M. Grote, M. John, A. Haas, B. Bramlage, L. M. Lckenstein, K. Jahn-Hofmann, F. Bauss, W. J. Cheng, R. Croasdale, K. Daub, S. Dill, E. Hoffmann, W. Lau, H. Burtscher, J. L. Ludtke, S. Metz, O. Mundigl, Z. C. Neal, W. Scheuer, J. Stracke, H. Herweijer, U. Brinkmann, *Mol. Ther.--Nucleic Acids* **2012**, *1*.
[243] S. Metz, A. K. Haas, K. Daub, R. Croasdale, J. Stracke, W. Lau, G. Georges, H.-P. Josel, S. Dziadek, K.-P. Hopfner, A. Lammens, W. Scheuer, E. Hoffmann, O. Mundigl, U. Brinkmann, *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 8194-8199.
[244] W. J. McBride, P. Zanzonico, R. M. Sharkey, C. Norén, H. Karacay, E. A. Rossi, M. J. Losman, P. Y. Brard, C. H. Chang, S. M. Larson, D. M. Goldenberg, *J. Nucl. Med.* **2006**, *47*, 1678-1688.
[245] D. M. Goldenberg, R. M. Sharkey, G. Paganelli, J. Barbet, J. F. Chatal, *J. Clin. Oncol.* **2006**, *24*, 823-834.
[246] J. M. Ledoussal, A. Gruazguyon, M. Martin, E. Gautherot, M. Delaage, J. Barbet, *Cancer Res.* **1990**, *50*, 3445-3452.
[247] M. E. Hahn, T. W. Muir, *Trends Biochem. Sci.* **2005**, *30*, 26-34.
[248] A. J. Bullous, C. M. A. Alonso, R. W. Boyle, *Photochem. Photobiol. Sci.* **2011**, *10*, 721-750.
[249] F. Kampmeier, M. Ribbert, T. Nachreiner, S. Dembski, F. Beaufils, A. Brecht, S. Barth, *Bioconjugate Chem.* **2009**, *20*, 1010-1015.
[250] J. R. Junutula, S. Bhakta, H. Raab, K. E. Ervin, C. Eigenbrot, R. Vandlen, R. H. Scheller, H. B. Lowman, *J. Immunol. Methods* **2008**, *332*, 41-52.
[251] S. K. E. Messerschmidt, A. Kolbe, D. Mueller, M. Knoll, J. Pleiss, R. E. Kontermann, *Bioconjugate Chem.* **2008**, *19*, 362-369.
[252] D. Beckett, E. Kovaleva, P. J. Schatz, *Protein Sci.* **1999**, *8*, 921-929.
[253] A. Tirat, F. Freuler, T. Stettler, L. M. Mayr, L. Leder, *Int. J. Biol. Macromol.* **2006**, *39*, 66-76.
[254] A. Keppler, M. Kindermann, S. Gendreizig, H. Pick, H. Vogel, K. Johnsson, *Methods* **2004**, *32*, 437-444.
[255] A. Gautier, A. Juillerat, C. Heinis, I. R. Correa, Jr., M. Kindermann, F. Beaufils, K. Johnsson, *Chemistry & Biology* **2008**, *15*, 128-136.
[256] T. Gronemeyer, G. Godin, K. Johnsson, *Curr. Opin. Biotechnol.* **2005**, *16*, 453-458.
[257] M. Kindermann, N. George, N. Johnsson, K. Johnsson, *J. Am. Chem. Soc.* **2003**, *125*, 7810-7811.
[258] W. Fischer, M. Calderon, A. Schulz, I. Andreou, M. Weber, R. Haag, *Bioconjugate Chemistry* **2010**, *21*, 1744-1752.
[259] S. Park, S. K. Lee, K. Y. Lee, *J. Controlled Release* **2011**, *152, Supplement 1*, e165-e166.
[260] A. Swami, A. Aggarwal, A. Pathak, S. Patnaik, P. Kumar, Y. Singh, K. C. Gupta, *Int. J. Pharm.* **2007**, *335*, 180-192.
[261] Y. Maeda, F. Pittella, T. Nomoto, H. Takemoto, N. Nishiyama, K. Miyata, K. Kataoka, *Macromol. Rapid Commun.* **2014**, *35*, 1211-1215.

8 List of Publications and Conference Contributions

8.1 List of Publications

[1] "Dendritic and lipid-based carriers for gene/siRNA delivery"

F. Sheikhi Mehrabadi, W. Fischer, R. Haag, *Curr. Opin. Solid State Mater. Sci.* **2013**, *16*, 310-322.

[2] "Multivalent dendritic polyglycerolamine with arginine and histidine end groups for efficient siRNA transfection"
F. Sheikhi Mehrabadi, H. Zeng, M. Johnson, C. Schlesener, Z. Guan, and R. Haag, *Beilstein J. Org. Chem.* **2015**, *11*, 763–772.

[3] "Optimized effective charge density using polyglycerol amines leads to strong and target specific knockdown efficacy"
Anna Maria Staedtler, Markus Hellmund, **Fatemeh Sheikhi Mehrabadi**, Bala N. S. Thota, Thomas M. Zollner, Markus Koch, Rainer Haag and Nicole Schmidt, *J. Mater. Chem. B*, **2015**, *3*, 8993-9000.

[4] "Structure-Activity Relationship Study of Dendritic Polyglycerolamine for Efficient siRNA Transfection"
F. Sheikhi Mehrabadi, O. Hirsch, R. Zeisig, P. Posocco, E. Laurini, S. Pricl, R. Haag, W. Kemmner and M. Calderón, *RSC Adv.* **2015**, *5*, 78760-78770.

[5] "Bispecific Antibodies for Targeted Delivery of Dendritic Polyglycerol (dPG) Prodrug Conjugates"
F. Sheikhi Mehrabadi, J. Adelmann, S. Gupta, S. Wedepohl, M. Calderón, U. Brinkmann and R. Haag, *Curr Cancer Drug Targets* **2016**, *16*, 1-11.

8.2 Conference Contributions

- 9th International Symposium on Polymer Therapeutics: From Laboratory to Clinical Practice, 2012, Valencia, Spain (Poster presentation)
- 4th International Symposium on Biomedical Applications of Dendrimers, 2014, Lugano, Switzerland (Poster presentation)
- 8th Molecular interactions workshop, 2012, Berlin, Germany (poster presentation)

9 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

10 Abbreviations

AAV	adeno-associated virus
ADC	antibody drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
AGO2	Argonaute 2
AGT	O^6-alkylguanine-DNA alkyltransferase
AMPA	bis-(3-aminopropyl) methylamine
ApoB	apolipoprotein B
BG	benzylguanine
bFGF	basic fibroblast growth factor
BP	boranophosphate
bp	base pair
bsAb	bispecific antibody
CD	cyclodextrin
CDC	complement-mediated cytotoxicity
CME	clathrin-mediated endocytosis
CDP	CD-based polymers
C_L	clearance rate
cRGD	cyclo-Arg-Gly-Asp
dsRNA	double-stranded RNA
DF	degree of functionlization
dPG	dendritic polyglycerol
EBOV	Ebola virus
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPR	enhanced permeation and retention
GSH	glutathione
HPMA	N-(2-hydroxypropyl) methacrylamide
Ig	Immunoglobulin
LMW-PEI	low molecular weight poly(ethyleneimine)
LNAs	locked nucleic acids

Abbreviations

LNP	lipid nanoparticle
mAbs	monoclonal antibody
miRNA	microRNA
mRNA	messenger RNA
siRNA	short interfering RNA
PD	pharmacodynamic
PEG	polyethylene glycol
pDNA	plasma DNA
PDI	polydispersity index
PEI	poly(ethylene imine)
PGA	poly-L-glutamic acid
PS	phosphorothioate
PVP	poly(vinylpyrolidone)
PEHA	Pentaethylenehexamine
RES	reticuloendothelial system
RISC	RNA induced silencing complex
RNAi	RNA interference
SARS	severe acute respiratory syndrome
siRNA	short interfering RNA
SNALP	stable nucleic acid-lipid particles
UNA	unlocked nucleic acids
VPF	vascular permeability factor
VEGF	vascular endothelial growth factor