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High Molecular Weight Polyglycerol based Nanocarriers for Gene Delivery

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2. Gutachter: Prof. Dr. Nan Ma

Disputation am Donnerstag, 10. September 2015

Für meine Eltern

Für meine Schwester

Für Lilo und Rudi

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Table of Contents

Abbreviations

Da	Dalton
DB	Degree of branching
DLS	Dynamic light scattering
DMM	Double-monomer methodology
DP	Degree of polymerization
GPC	Gel permeation chromatography
hPG	Hyperbranched polyglycerol
hPG amine	Hyperbranched polyglycerol amine
nPG amine	Polyglycerol amine nanogels
Indices n and m	Indices for natural numbers
IR	Infrared spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
PAMAM	Poly (amido amine)
PDI	Polydispersity
PEG	Poly (ethylene glycol)
PEI	Poly (ethylene imin)
PLL	Poly (L-lysin)
PTP	Proton-transfer polymerization
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA- interference
dsRNA	double stranded RNA
miRNA	Micro RNA
piRNA	Piwi-interacting RNA
siRNA	Small interfering RNA
ROMBP	Ring opening multi branching polymerization
SMA	Slow monomer addition
THP	Tetrahydropyrane

1 Introduction

1.1 Gene therapy

The field of gene therapy[1] was founded in the 60's due to the elucidation of the DNA structure. The concept of gene delivery, the exchange of damaged DNA with new foreign DNA was developed by T. Friedmann and R. Roblin,[1] both shaped the term *"gene therapy"*. But only advanced macromolecular methods promote the field using inventions like recombinant DNA technology (developed in the 80's on retroviruses[2]). Since then, more than 1800 clinical trials on gene therapy have been evaluated or are in the process of testing.[3] Besides the standard method of gene substitution, alternatives like the "repairing" of genetic material *in situ* with specialized nucleic acids was developed.[4]

1.1.1 RNA-interference (RNAi)

Another approach is the inhibition of gene expression by ribonucleic acid (RNA).The RNA-interference (RNAi) referred to the mechanism of the sequence specific inhibition, which was discovered by Fire *et al.*[5] and awarded with the Nobel Prize in 2006 together with C. Mello. Elbashir *et al.* finally postulated the gene silencing in human cell lines with small interfering RNA (siRNA).[6] Nowadays, RNAi is known to combine several mechanistically similar processes, in which small non-coded RNA molecules can regulate sequence specific genes.[7] Thereby, three different main categories are used: siRNA, *"micro RNA"* (miRNA) and *"piwi-interacting RNA"* (piRNA).[8] To date, siRNA and miRNA are the most commonly used RNAs, but their differentiation gets more and more problematic. At first, it was mentioned that miRNA is an endogenic, target specific expressed product of its own genome with incomplete double stranded character and siRNA, in particular, exogenous origin cut out of long complete double stranded RNA, derived from viruses or transgenic trigger.[8,9] But at the same time, it was shown that miRNA and siRNA are similar in their size (21 to 23 nucleotides), sequence specific inhibit function and their interaction with the DICER enzyme to be cut out of their precursor, and the Argonaut to support their silencing function,[9,10] and so consequently both are part of the si- /miRNA mediated RNAi.

[1] *"Gene therapy can be defined as the treatment of human disease by the transfer of genetic material into specific cells of the patient. The transfer of genetic material is defined as gene delivery, the technique to induce new foreign genetic material into the host or host cell."* [29,30]

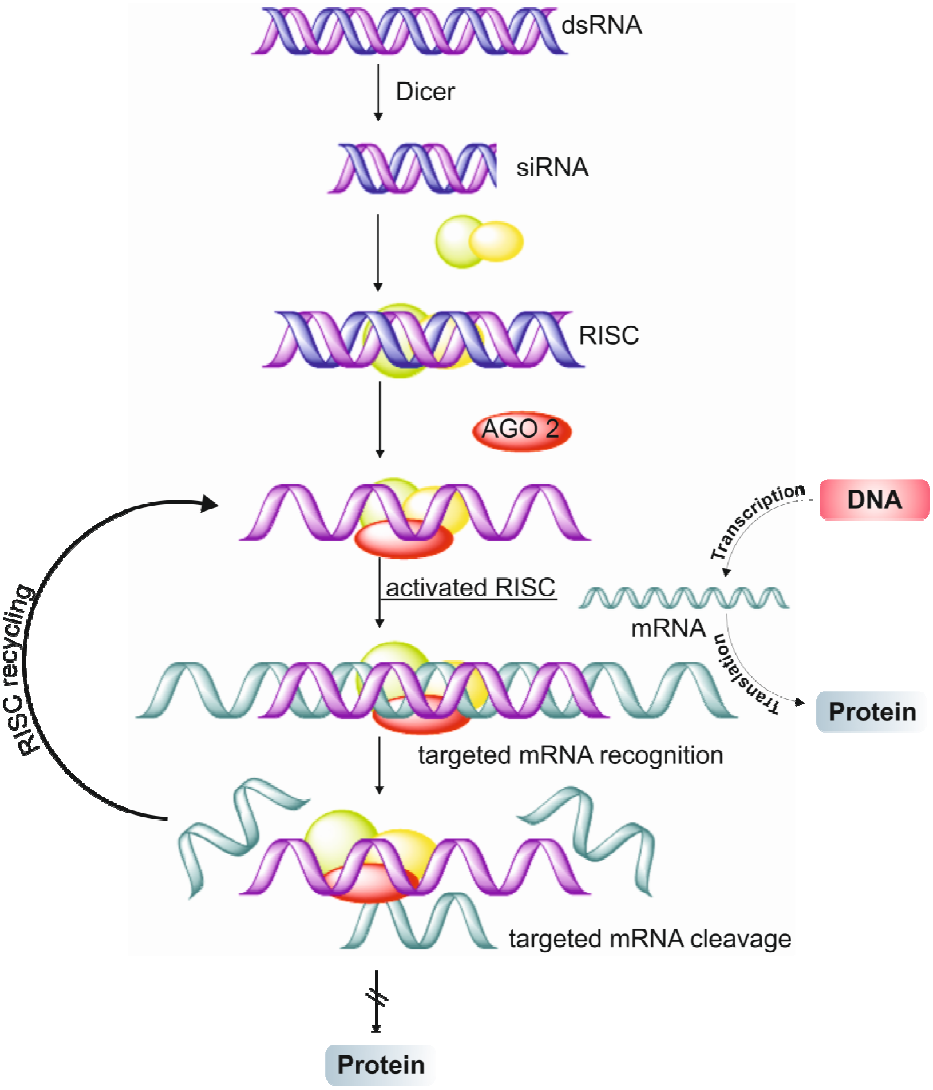


Figure 1. Inhibition of gene expression using siRNA mediated RNAi. This figure is used with the permission of Elsevier Ltd. and the author F. Sheikhi Mehrabadi.[11]

RNAi plays a fundamental role in various eukaryotic functions, with a focal point on the regulation of endogenic protein coded gene expression.[12] In principle, all these effects caused by small RNAs are inhibiting and are based on gene expression and –controlling. That's why the corresponding method is called "*RNA-silencing"*.[8] The mechanism of the inhibition of gene expression using siRNA mediated RNAi is shown in Figure 1. The DICER enzyme (Figure 2) litigates the double stranded RNA (dsRNA) with its PAZ subunit on the favorable 3'end position with a two nucleotides transition. The distance of the PAZ subunit to the RNase IIIa and IIIb, both are also located in the DICER enzyme (Figure 2), thereby controls the length of the resulting siRNA, normally 20-30 base pairs.[13] After this catalytically splitting of the dsRNA into smaller siRNA strings, the *"RISC-loading complex"* (RLC) incorporated these siRNA strings in the multiprotein complex, *"RNA-induced silencing complex"* (RISC).[14] Within the RISC and further divided into three main domains, the *"mid domain"* to bind siRNA at the 5'-monophosphat unit, the *"PAZ domain"*, similar in function to

the one of the DICER enzyme and the *"piwi domain"*, mainly responsible for the separation of the sense and antisense string, the subunit *"Argonaut 2"* splits the phosphor diester bond of the mRNA during the siRNA mediated RNAi. In addition, Argonaut 2 separates the "*guide"*-(thermodynamically less stable at the 5'end position, remains in the RISC) from the "*passenger"* string.[15] The efficient siRNA mediated RNAi was defined with a resulting siRNA of a length of 21-23 nucleotides with a guanine/cytosine content of less than 50%. More detailed, the 5' end of the antisense string should have an adenine/uracil base pair and the sense string a guanine/cytosine base pair. Moreover, the antisense string needs five adenine/uracil units in the last third and there should not be more than nine guanine/cytosine base pairs in series.[16]

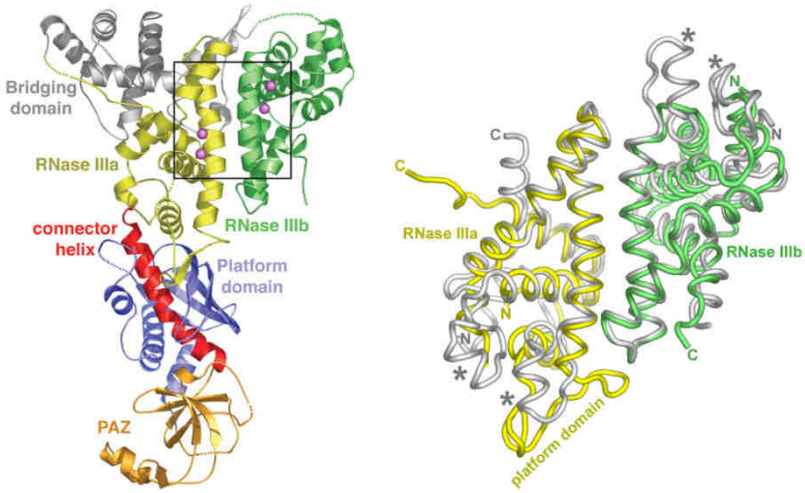


Figure 2. Schematic illustration of the DICER enzyme and the RNase IIIa and RNase IIIb subunits. These figures are used with the permission of the American Association for the Advancement of Science and the author I. J. MacRae[17]

To improve the efficiency of the siRNA mediated RNAi, there is a need of a highly suitable gene delivery vector. Many skills have to be addressed: it has to condense the siRNA efficiently and to protect against degradation, to overcome several intra-/extra cellular barriers, induce cell entrance or the escape from the endosomal vesicle.[18–20] Besides that, the biocompatibility *in vivo* of a nanocarrier has to be defined by its physicochemical properties. Nel *et al.* visualized these properties in a 3D diagram, the rigid core size, the hydrophobicity and the zeta-potential (Figure 3).[21] With it, the author could demonstrate that cationic particles with a high surface reactivity are more toxic than hydrophobic. Furthermore, these hydrophobic particles are cleared faster and safer by the mononuclear phagocyte system, but they have to be within the range up to 10 nm in diameter, otherwise they have to be biodegradable.

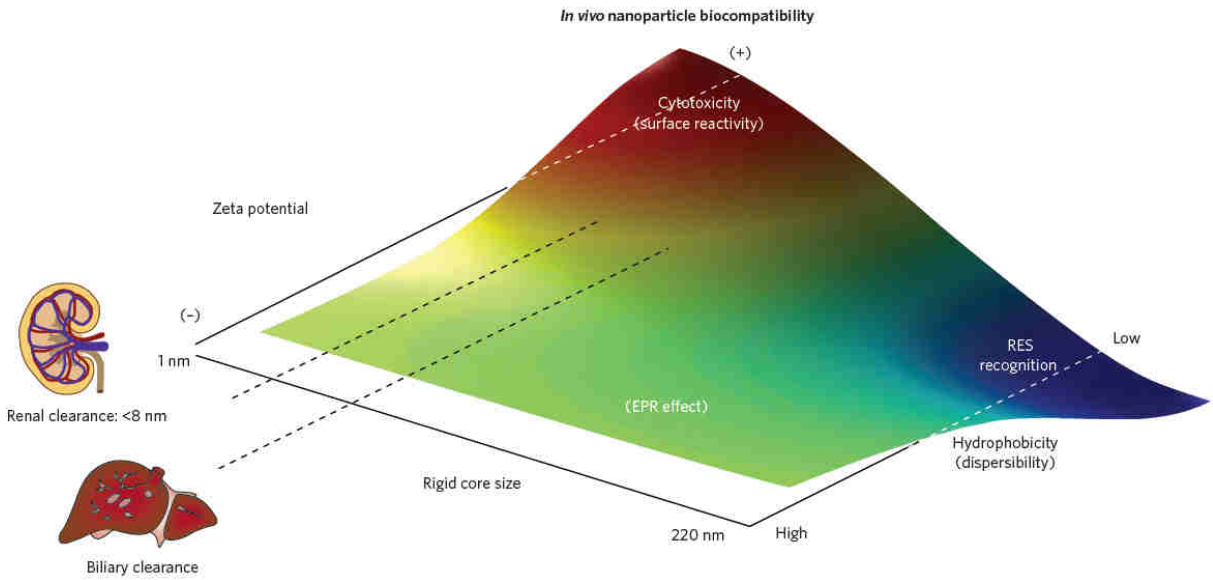


Figure 3. Physical characteristics of nanoparticles determine *in vivo* biocompatibility. The red colored area represents toxicity and the bluish high compatibility *in vivo*. This figure was reproduced with the permission of Macmillan publishers limited and the author A. E. Nel.[21]

Interestingly, neutral or less positively charged particles showed the highest "e*nhanced permeation and retention effect"* (EPR-Effect).[20] As known from literature, larger particles can selectively reach the site of inflammatory response by passive targeting due to this EPR-Effect. The term EPR effect was first described by Maeda *et al.* in the 80's,[22] leading to an improved passive accumulation of macromolecules in the tumor issue. Typically, the cellular plasma membrane serves as a barrier which occludes the transport of molecules based on the molecular weight, size, polarity and charge of the nanocarrier. In comparison to the normal tissue, tumor cells exhibit an enhanced leaking which results in an elevated permeability to nanocarriers. Interestingly, most nanocarriers with an effective EPR effect have a neutral surface charge and a bigger size than 10 nm. Theoretically, any high molecular weight nanocarrier, which can act as a water-soluble transporter for drugs, polymeric drugs or liposomes should display passive targeting. However, the degree of accumulation in the tumor is depending on the attributes of the nanocarrier (Size, molecular weight, charge and hydrophilic character).[23,24] Notably, the existence of this predicted EPR effect has been experimentally confirmed by M. Thanou and R. Duncan.[25]

1.1.2 Polyplex formation for gene delivery

Gene delivery constitutes one of the major procedures demonstrating the potential as well as the difficulties of the transport of genetic material into the patient. Various cationic lipids and cationic polymers were investigated for many different biomedical applications by forming condensed complexes with negatively charged genetic material through electrostatic

interactions.[11,26–28] This complexation is known as "*polyplex formation*" (Figure 4) and protects the negatively charged genetic material from degradation and facilitates its cellular uptake and intracellular trafficking into the nucleus.[29,30] Furthermore, gene delivery has been proposed as a promising strategy for therapeutic issues, for instance, cancer[31] or DNA vaccination.[32]

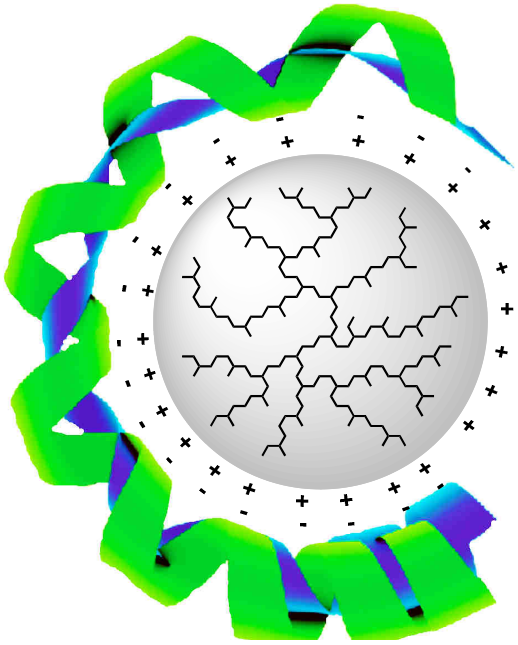


Figure 4. Schematic illustration of a polyplex formation between a DNA string and a hyperbranched cationic polymer, such as poly (ethylene imine) (PEI).

Naked DNA molecules are not taken up efficiently by the cells, because of their large size and their hydrophobic nature due to the negatively charged phosphate groups. In addition, DNA molecules are very susceptible to nuclease-mediated degradation.[33] *"On their way from the test tube to the cellular membrane"*,[29] at first, non-viral gene delivery vectors have to overcome the extracellular barriers, like gene packing, the shielding of the naked DNA against degradation, serum stability and cell specific targeting.[28,34] Secondly, intracellular barriers, like endolyosomal escape, nuclear localization, and the transport through the cytoplasm have to be addressed.[29,35] Various polymers were mentioned to be highly efficient for the delivery of genetic material into the cell through electrostatic interactions between the negatively charged genetic material and the positively charged polymer. The surface charge of a polyplex or lipoplex (Polyplex in the case of a lipid or liposome and genetic material) depends on the nature of the polymer and the condensing material, such as DNA. Classical studies of colloid behavior assumed that particles of similar charge profiles retain an electrostatic repulsion, which makes them stable against aggregation since their strength is higher than their Van-der-Waals force.[36] However, these positively charged particles easily aggregate as their concentration increases. Suggesting that they do not comply with the

classical model, it has to be modified by use of complex models such as bridging the gap between polymers with polymeric loops.[37] In addition, positive charge densities improve endosomal escape due to the *"proton-sponge"* effect,[38] because of their ability to buffer the pH-values during cell entry. Poly(ethylene imine) (PEI) as well as poly(amido amine) (PAMAM), having a high number of secondary and tertiary amines, is known to force the ATPase to pump protons into the endosomal vesicle causing osmotic swelling and rupture of the endosomal membrane. Therefore, the polyplex is released into the cytoplasm.[39] Besides the proton sponge effect, the so called *"flip-flop"* mechanism was proposed.[40] This model is based on the electrostatic interactions between the cationic lipids and the endosomal membrane. The local destabilization of the membrane is thereby caused by displacement of anionic lipids from the cytoplasm-facing monolayer of the endosomal membrane. The formation of a neutral ion pair between anionic lipids present in the endosomal membrane and the cationic lipids of the vector will then cause subsequent decomplexation of the DNA and finally its release into the cytoplasm.[41] Recent research efforts are focused to increase the efficacy and to improve the understanding of gene delivery processes. However, the gene delivery process is complex and therefore the interpretation of results is not always straightforward, and libraries of structurally diverse transfection agents have provided insights into which parameter influence non-viral polymer mediated gene delivery.[42] One of them is the charge density of the nanocarrier, a key parameter for successful binding of genetic material, interaction with the cell and cell surface, and to cross extra-/intracellular barriers during cellular uptake. However, increasing the charge of the polymer can improve the transfection efficacy but may also enhance cytotoxicity.[43,44] To date, systemic administration has usually resulted in a toxic response in both cases, lipids and polymers, related to the positive charge density, which makes them incompatible within clinical trials.

1.1.3 Polyvalent effect and charge interactions

The interaction of the charge and the polyvalent[2] effect can be described with the strength of the interaction between two systems, for instance, cells or viruses. Fasting *et al.* concluded that the synergy between the polyvalent interaction of the functional groups on the particle and the strength of the interaction of the particle plays a major role for the application of polymer based nanocarriers *in vivo*.[45] To understand the importance and advantage of a polyvalent binding compared to a monovalent interaction, the limitations of a monovalent system will be firstly mentioned. Monovalent interactions are based on a one to one ratio, meaning that a monovalent ligand can connect to either a mono- or a polyvalent analog.

[2] In this thesis, the difference between a multivalent or polyvalent interaction will not be considered, as mentioned by Fasting *et al.* [45] Normally, the word polyvalent is used to describe a huge number of interactions, which is true for the polymeric scaffolds used.

Thereby the charge of a monovalent system is limited to the one group as well as its binding strength. To increase the binding affinity in case of a mono- and a polyvalent interaction, a huge amount of monovalent systems is needed. Especially during *in vivo* applications, like shielding a virus, the local concentration of the monovalent binder has to be extremely high to inhibit all binding sites, which limits its efficiency.

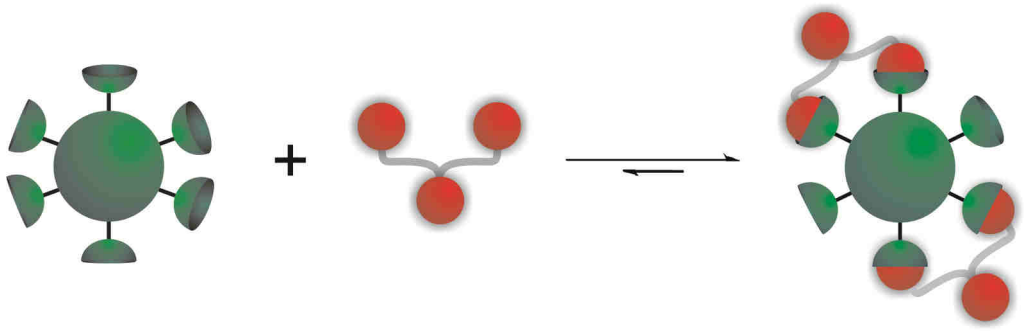


Figure 5. Schematic illustration of polyvalent interactions between a multifunctional receptor and a multivalent ligand.

As shown in Figure 5, a polyvalent interaction can take place between a multifunctional receptor and a multivalent ligand. Using a spherical polyvalent scaffold, like the hPG with the advantage that 60% of the chargeable groups are located on the surface,[18] its polyvalent effect can be selectively adjusted by the size of the polymer. The size difference of a 10 kDa hPG (ca. 81 functional groups on the surface) and a 100 kDa hPG (ca. 810 functional groups on the surface) is improving the possibility of an interaction by the factor of ten. But as described above, not all functional groups are located on the surface or are not arranged in the contact area. Consequently, another important factor plays a crucial role for adjusting the polyvalent effect, the charge density. In the case of a 100 kDa hPG scaffold 540 functional groups are located as defects inside the structure (10 kDa = 54). Not the entire charge density is used and therefore an effective charge density is generated. The effective charge density describes the remaining charge after a polyvalent interaction between two particles and can be directly correlated to the sum of all functional groups per surface area. For instance, this effect enables an interaction of the particle with a cellular membrane even after previous complexation of genetic material. This major advantage of a spherical polyvalent system enhances the application *in vivo* dramatically. The effective charge density in relation to the multiplicity of the chargeable groups plays a crucial role in biomedical applications.

1.2 Non-viral gene delivery vectors

Basically, gene delivery methods are categorized in three main classes: viral and non-viral vectors or physical/mechanical techniques.[46] Physical and mechanical implementation is based on an artificial permeation of the cellular membrane. Most commonly, the microinjection is used to transfer directly oligonucleotides into the cell. This method was developed by Capecci *et al.* in the 80's.[47] Besides, the method electroporation is used *in vitro* to destabilize the cellular membrane using locally generated electric fields[46] or *in vivo* the so called *"Particle bombardment"* realized with the *gene gun* is used to transfer exogenic gene material, for instance into the skin.[48] Furthermore techniques based on ultrasound, magnetic fields or laser irradiation are known.[46] Even though they are efficient in locally narrowed transfection, all these methods can cause cell or tissue damage or bleedings due to the mechanical injection.[49] Instead, viral vectors – adenovirus, adeno-associated viruses or retro-/lent viral vectors – are highly developed and can use the cellular machineries to enter the cell and start their own replication.[50] But they often exhibit immunogenicity and mutagenicity or suffer from expensive and laborious production in large quantities.[34,50] Conclusively, the research was more concentrated on non-viral vectors for gene delivery including peptides, lipids, proteins and polymeric scaffolds.[51] For instance, in the field of lipids or lipid based architectures, different aspects were studied, like head groups,[52] polymer based lipids[53,54] and the charge dependend behavior of lipids *in vitro*.[55,56] All these systems are suitable due to their high potential of specific adaptation, cost-saving large-scale production and improved capacity for therapeutic genes.[29] However, non-viral gene delivery scaffolds showed universal significant reduced transfection efficacy due to complications in facing the numerous extra- and intracellular barriers compared to the viral ones, but they are advantageous in view of their biocompatibility profile. Nonetheless, fine-tuning all these cationic, non-viral scaffolds would revolutionize gene therapy, as the ability to fully control the charge excess is highly challenging.

1.2.1 Poly (ethylene imine) and poly (amido amine)

Poly (ethylene imine) (PEI) and poly (amido amine) (PAMAM) have been reported for their good DNA transfection.[20,41,57–61] Thereby, PEI has remained the 'gold standard' since its discovery in 1995.[39] PEI is the most efficient polymer based transfection agent *in vitro*. PAMAM dendrimers are also known to be highly efficient.[62] Both polymers are still used as a positive control in many disease models, but remaining positive charge after polyplex formation can lead to apoptosis and necrosis of cells due to destabilization of the cellular membrane.[63,64] Furthermore, with the increase in size the total amount of positively charged amine groups increases as well due to the high amine group content in the backbone of both

polymers. Therefore, the efficient adjustment of charge is not possible as this is an invariable property of each polymer.

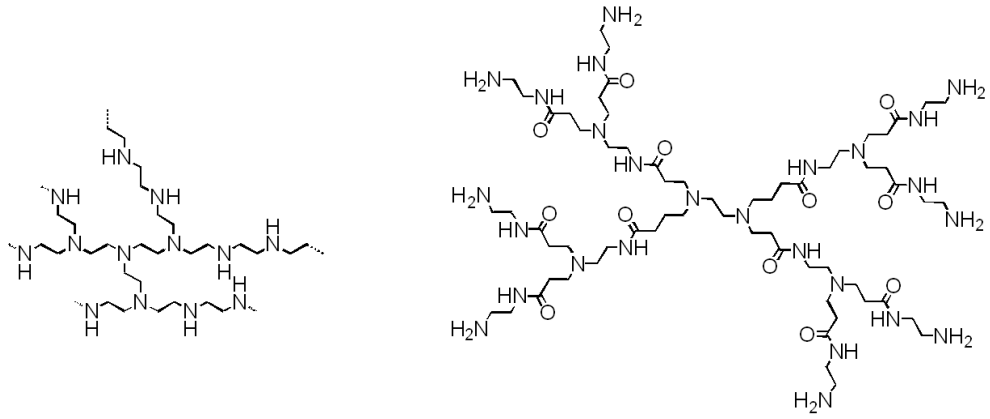


Figure 6. Schematic illustration of PEI and PAMAM.

As possible solutions to this problem, lower molecular weight PEIs were intensively studied to avoid the positive charge excess or the fine-tuning of the charge density by employing methods such as the PEGylation and acetylation were investigated.[65] Both effects describe the introduction of opposite or neutral loaded polymers/functional groups, which covers up/hide the normally high charge density of the original polymer. As already mentioned above, for instance, these PEG/PEI-derivates were broadly investigated in the last decades.[33,66–69] It turned out that the synergy between the sizes of both polymers plays a crucial role in the final effective charge density of the core-shell architectures. It was shown that high packed core-shell architectures with low molecular weight PEG are more efficient than less packed ones with high molecular weight PEGs.

Another crucial parameter is the flexibility - in case of spherical structures also called the degree of branching (DB) - giving a hint towards accessibility of the functional groups as already mentioned above. With an increasing DB the ability of electrostatic interactions of the 'inner core' groups with the counterparts will be decreased. Due to lower capability to penetrate the outer sphere of the neighboring compounds, the 'inner core' functional groups have no impact on electrostatic interactions. However, these inaccessible groups have a direct impact to the effective charge density. For instance, PAMAM possesses several of different amine groups in its globular structure. Thereby, the DB determines exactly the ratio of primary, secondary and tertiary amines in the structure and their influence on endosomal escape and cytotoxicity. It was reported that PAMAMs with similar molecular weights and structures, but varying DB, showed divergent behavior in polyplex formation.[70–72] It was found that with increase of branching, the cytotoxicity decreases, due to the fact that the hydrodynamic size is reduced and therefore the effective charge density could be easier

covered up resulting in a higher binding strength between DNA and polymer. Additionally, the influence of charge in combination with different PAMAM dendrimer generations as well as head group modifications was intensively studied for *in vitro*.[73–76] One of the major advantages of all these PAMAM dendrimers is their well-defined structure and polyvalent features, which increases with generation. It turned out that [G4/G5] dendrimers have an optimized charge profile for gene delivery.[77] PAMAM dendrimers are investigated for cancer treatment, as nanocarriers for delivery of imaging agents, drug components and therapeutic agents.[78,79] Their non-toxic behavior and their biocompatibility in biological systems enable their use as chemotherapeutic drug delivery systems. However, the major disadvantage is the possibility of retro-Michael addition which extremely limits their shelf-life under ambient conditions.[80]

1.2.2 Alternatives to PEI and PAMAM

In the case of poly L-lysine (PLL), the advantage for *in vivo* studies is the biodegradable nature and that low molecular weight structures cannot form stable polyplexes. At least a molecular weight of more than 3 kDa is necessary for sufficient charge density to build stable polyplexes with DNA and higher molecular weight PLL (≥200 kDa) are even more suitable for gene delivery.[81] Similar to PLL, also chitosan is a biodegradable and biocompatible linear amino-polysaccharide synthesized by using 1,4-linked N-acetyl-D-glucosamine and D-glucosamine subunits. Its ability to complex genetic material for stable polyplex formation with sizes between 20-500 nm depending on their molecular weight and degree of deacetylation was already reported as well as its cationic polyelectrolyte nature.[82,83] Through their charge density profiles, the successful protection against DNase degredation is as efficient as compared to PEI, but the biocompatibility is significantly better. Consequently, chitosan derivates were intensively studied as an alternative to PEI.[84] Kiang *et al.* reported that decreasing the degree of deacetylation and thus changing the charge density of chitosan results in a decrease of overall luciferase expression levels.[85]

In conclusion, the optimization of non-viral gene delivery vectors remains a challenging task for *in vivo* application. Yet as the numerous studies demonstrate there is a plethora of efforts on increasing the efficacy of gene transporters by only adjusting the effective charge density and flexibility with respect to size.

1.3 The scaffold hyperbranched polyglycerol

Polymer based nanocarrier[3] are increasingly recognized as universal scaffolds for biomedical applications. Among them, linear or branched poly(ethylene glycol)s (PEG) have been well-established as standard polymers for clinical prodrug conjugation, the so called *"PEGylation"*. Since its discovery, this technology is used, for instance, to improve the circulation time of liposomes in blood[86] or to design PEGylated products commonly used in laxatives (PEG 3350), skin creams, antifoaming agent or as enzyme conjugates, like Oncospar® (PEGylated L-asparaginase) and Adagen® (PEGylated bovine adenosine deaminase). Furthermore, linear, dendritic or star-like PEGylated architectures were investigated to control size, charge and surface functionality.[87,88] Thus, PEG and PEGylated scaffolds are the most commonly used polymers and represent the gold standard in the field of prodrug conjugation. However, due to unexpected changes in the pharmacokinetics' profile, toxic side products and potential accumulation in the body during administration, the named products have been critically addressed.[89] As these stated safety issues dictated alternatives to the commonly utilized PEG, they have been developed.

One of the most promising alternatives is the polyglycerol, especially the hyperbranched polyglycerol (hPG) scaffold. Polyglycerol to PEG based structures gained high relevance over the last decades, mainly due to their advantageous polyether backbone, but furthermore because of their well-defined structures, their high biocompatibility and their ability to adapt special requirements as needed in a huge variety of biomedical applications. Depending on the synthetic strategy applied, the polyglycerol scaffold can differ from linear to hyperbranched or even more defined structures (Figure 7). For instance, by protecting the primary hydroxyl group on the monomer glycidol, branching reactions can be easily prevented and linear, comb-like or star-like structures can be designed. Recently, Frey and coworkers reviewed these different synthetic pathways to gain linear polyglycerol based architectures.[90] In fact, oligoesters of linear polyglycerol with a degree of polymerization (DP) up to 10 are used as food and pharmaceutical additives since the late 90's.[89] Haag and coworkers could demonstrate that the antifouling properties of linear polyglycerol scaffolds are comparable to the one of PEGylated structures and that the adsorption of proteins and cells significantly decreases with the increase of the DP.[91,92] In contrast to their linear counterparts, the lack of entanglements due to the globular structure of hPG leads to well-defined structures for biomedical applications.

[3] *"The term "nanocarrier" is used to describe a hybrid multifunctional system with a size typically ranging between 1-200 nm which may deliver the bioactive agent at the target site with improved therapeutic activity over the free form of bioactive agent. To date, they are also involved in long circulating liposomes, polymeric prodrug conjugates, polymeric micelle, nano/microgels, and nanocomplexes."* [20]

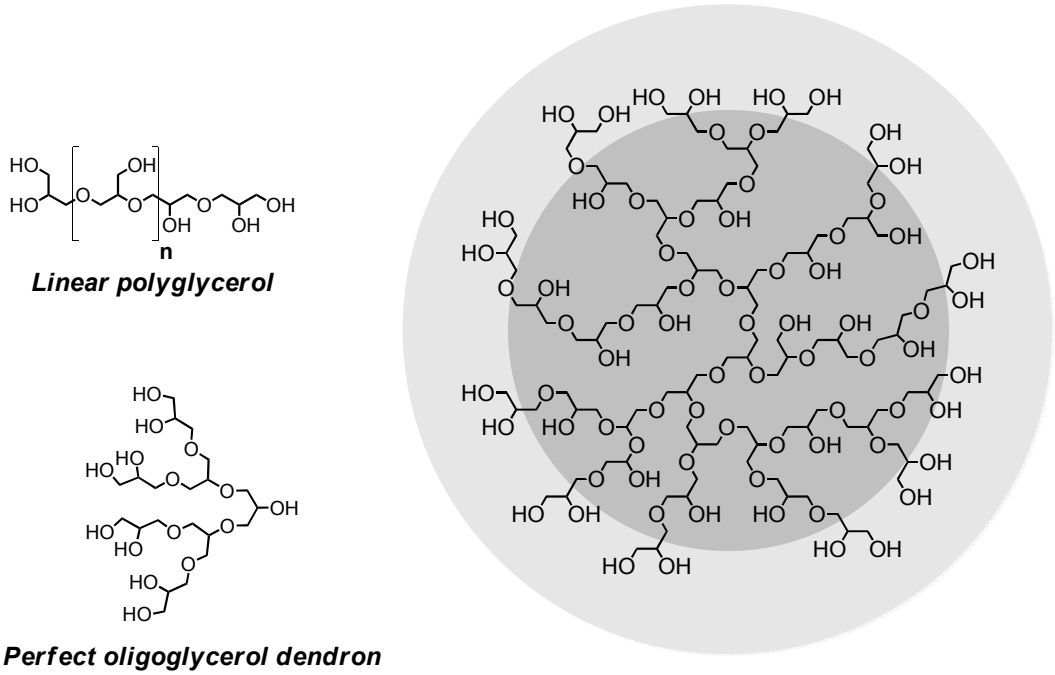
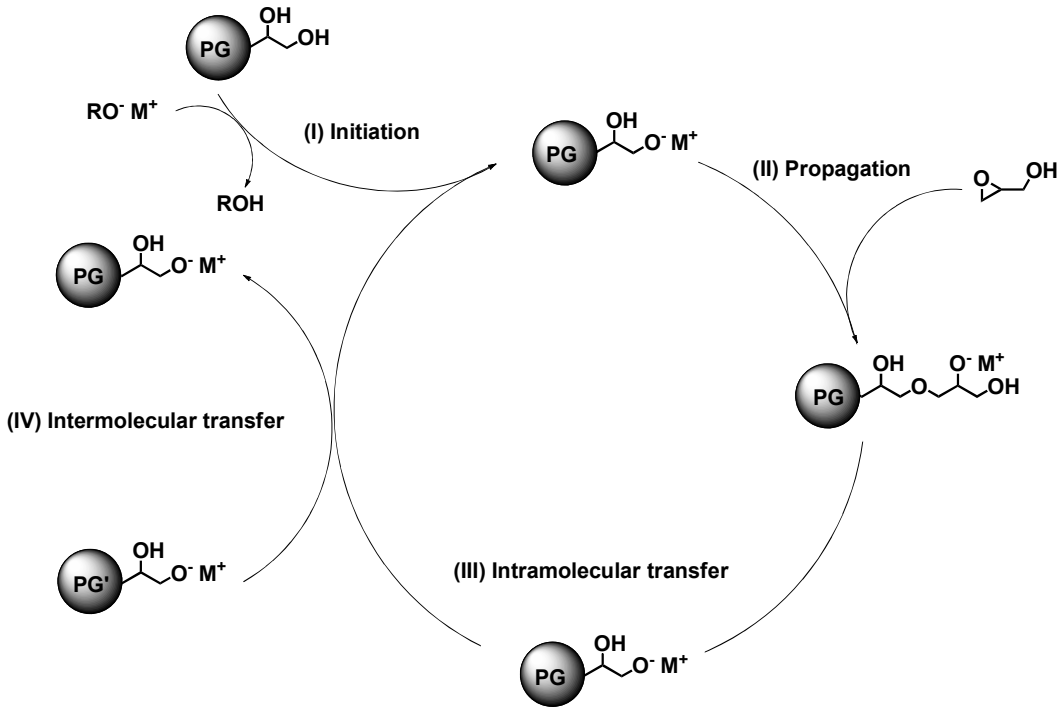


Figure 7. Schematic illustrated structural difference of linear, hyperbranched and perfect dendritic polyglycerol.

1.3.1 HPG – Design and history of the synthesis

Since the late 30's, *"polyhydric alcohol – polybasic acid reaction*",[93] was reported as a first synthesis generating hyperbranched polymers. Thereby the authors claimed; *"The tendency of the organic chemist to discard the tarry residues or resinous products of a reaction and to explain them away by merely mentioning their formation is unfortunate. It is quite probable that in the study of resin formation many new ideas of space chemistry will be brought forth which, although not revolutionary, will simplify our concepts of materials regarded as complex and at the same time bring about a better correlation between organic chemistry, colloidal phenomena and statistical mechanics."*[93] Their described reaction, classified as an A_nB_m type, between the monomers tartaric acid (A_2B_2) and glycerol (B_3) resulted in a series of polymeric products established in the industry. About 10 years later the authors published further studies on that type of reaction, which later on were combined as *"double-monomer methodology (DMM)*".[94,95] Almost at the same time, Flory published a first calculation on the incorporation of tri- and tetra-functional monomers and their effect on the molecular weight distribution.[96,97] This idea of three-dimensional polymeric scaffolds was finally reported in 1952, when Flory presented his concepts on *"degree of branching (DB)"* and *"highly branched architectures"*.[98] Furthermore, the suggested poly-condensation pathway to synthesize hyperbranched polymers using an AB_n-type monomer ($n \geq 2$) was realized by Kricheldorf and coworkers.[99] They compared the polymerization of an AB_n-Monomer or an

A_n- + B_m Monomer. Finally in the late 90's the term *"hyperbranched polymer"* was introduced by Kim and Webster.[100]



Schema 1. Schematic illustration of the ROMBP based on the reported mechanism by Sunder and coworkers.[101] The mechanism is divided into four steps; (I) Initiation, (II) Propagation, (III) Intramolecular transfer, and (IV) Intermolecular transfer.

The history on the polymerization of hyperbranched polyglycerol started in the late 60's, when Sandler and Berg reported the first synthesis using glycidol, an AB_2-type monomer having an epoxide group and a primary hydroxyl group, with various catalysts.[102] Depending on the catalyst, they explored different molecular weights of the final hPG, for example, using NaOMe as well as pyridine in catalytic amounts, molecular weights lower than 500 g mol^{-1} were reached. About 20 years later, in the late 90's Vandenberg reported the anionic polymerization of glycidol using KOH and potassium *tert*-butoxide (KO^tBu) as initiators getting an average molecular weight of approximate 2000 g mol^{-1}.[103] They assumed that the mechanism of the polymerization of glycidol is based on a so called *"proton-transfer polymerization (PTP)"*.[104] In the late 20th century, Frey and Multhaupt investigated the *"ring opening multi branching polymerization (ROMBP,* Schema 1*)"* of glycidol,[101,105,106] which is still in use nowadays. First of all and due to the topological developments of Frey and coworkers on AB_n-type monomers (n≥2),[107] the *"slow monomer addition (SMA)"* technique was investigated.[108] Considering the relation between SMA and the DB/molecular weight, hPGs could produce on a large scale.[105,106]

Fuelled by an increasing importance of the hPG scaffolds over the past years, further investigations were conducted giving rise to a growing variety of hPG based nanocarriers. Especially, Frey, Haag and coworkers reported various possible monomers and initiators for the ROMBP[101,109] as well as post-modifications on the hPG core, like negatively charged hPG-sulfates as inhibitors for inflammation.[110] Additionally, hPG scaffolds have been evaluated in diverse studies, for instance, the size dependent uptake of hPGs,[111] the receptor mediated uptake of low molecular weight hPGs,[112] the solvent dependent polymerization of high molecular weight hPG[113] or biocompatibility studies of different hPGs synthesized in the research group of D. Brooks *et al.*[114–116] Furthermore, a growing database supports the use of hPGs for biomedical applications.[20,60,89,95,117–120] Conclusively, the high density of functional groups on the surface, the low viscosity, the straight forward synthesis and the possibility to scale-up the reaction, its bioinertness and biocompatibility in vivo made hPG scaffolds useful in a broad range.

1.3.2 Micro- and nanogels

As a large scale alternative with diameters higher than 50 nm, the classes of nano- and microgels was developed in recent years.[119,121,122] Functionalized nanogels could be optimized for gene delivery by increasing their polyvalent effect[123] or could be loaded with insoluble drugs as pharmaceutical carriers.[124] Additionally, specialized nanogels were intensively studied as tumor targeting nanogels[125] or non-fouling biomedical device coatings based on hydrogels.[126] The structural dependence of nanogels and their polyvalent effect was intensively studied for siRNA delivery[127] and tunable encapsulation of proteins and charge dependent cellular uptake could be demonstrated.[128,129] Sisson and Haag showed the inherent properties of polyglycerol based nanogels having a size between 20 nm up to several micrometers.[130] For instance, polyglycerol based nanogels with sizes between 25 and 350 nm can rapidly internalize into cells, with a preferred localization nearby the nucleus. In addition, disulfide containing polyglycerol based nanogels were found to be highly biocompatibility and degradable[131] and thermo-responsive polyglycerol based nanogels show multiple stimuli response with sizes between 50 and 200 nm. The incorporation of polyglycerol as cross linking agent enhanced the water solubility of the nanogels, improve its biocompatibility profile and allowed a fine tuning of the thermo-responsive profile regarding the size of the nanogels in solutions.[132]

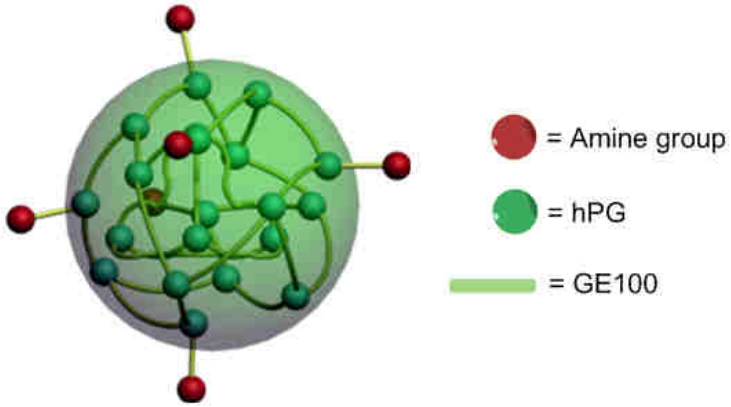


Figure 8. Schematic illustration of a nPG amine based on the recently published work by Zhou *et al.*[133]

Recently, Zhou *et al.* reported on polyglycerol-based nanogels synthesized by mini emulsion technique.[134] These nanogels are a special case of high molecular weight polymers with characteristics of the dendritic polyglycerol and cross-linked hydrogels, the combination of a tunable size of 20-200 nm and the high density of surface functionality. Polyglycerol based nanogel show a high Young's modulus, which is a sign of a rigid core structure and were investigated in the last years concerning stiffness and size. Zhou *et al.* were able to synthesize a series of defined nanogels in a swollen diameter size of 80-120 nm.[133] These compounds were obtained by acid-catalyzed epoxide-opening polymerization of glycidol in mini emulsion. The properties of these nanogels, i.e. size, degree of branching and viscosity were studied and can be controlled by varying the functionalities of the monomers and cross linkers. The effect of charge density on these nanogels as potential nanocarrier for genetic material was evaluated by comparing their cellular uptake behavior.[129]

1.4 Polyglycerol amine for gene delivery

As mentioned above, hPG is a promising candidate because of its high biocompatibility, a globular structure with a high density of hydroxyl groups, biological inertness *in vivo*, tunable end group functionalities, and inertness to non-specific interactions with biological environments.[18,20,72] Therefore the scaffold is an ideal candidate for further modifications.

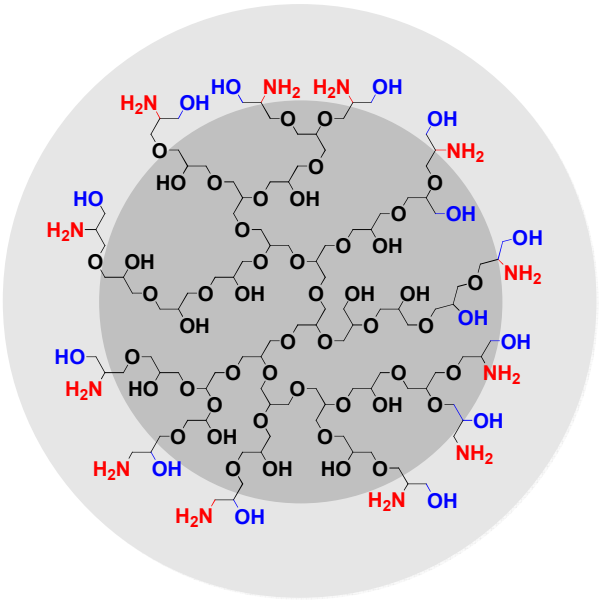


Figure 9. Schematic presentation of a moderate loaded polyglycerol amine scaffold.

Combining the polyglycerol scaffold with the advantages of PEI or PAMAM, the high density of amine groups on the surface, the hPG amine was envisioned. Thus polymer with a polyether backbone and the possibility to optimize the effective charge density by controlling the amine loading on the surface would potentially be a highly suitable candidate for gene delivery. In our group, first biological evaluations were performed on low molecular weight hPGs with different oligoamine core-shell type structures. These post modification allows an easy control of the toxicity/transfection ratio.[135,136] Fischer *et al.* could prove that it is possible to obtain a better transfection/toxicity ratio by fine tuning the amine content on the surface or shell, respectively. Recently, they reported the synthesis of only one hPG amine, the so called *"PG-NH$_2$"*, with an average MW of 10 kDa and primary amines in a favorable 1,2-arrangement. This candidate has a high amine density of 90% of the total functional groups. The PG-NH$_2$ showed the best binding affinity towards gene fragments based on an ethidium bromide displacement assay of all core-multi-shell-polyglycerol amines. The charge/pH behavior and the favorable 1,2-amine positions, results in a low toxicity profile and a good transfection efficacy. In comparison to silencing efficiency and toxic side effects with PEI derivatives, the polyglycerol amine architecture showed a better toxicity profile.[135–139] However, a major drawback of this special hPG amine was the fast clearance in vivo, which limits its applications. With the biological evaluation of Brooks and coworker concerning high molecular weights hPGs, showing a similar or even better biocompatibility profile than PEG with a MW ranging from 4.2 kDa to 670 kDa.[140–142] and with the knowledge that particles with hydrodynamic sizes of more than 10 nm can use the EPR-effect, higher molecular weight hPG amines have to be developed as their polyglycerol analogs exhibit longer circulation half life times in the blood stream.[115,116,140]

The combination of a high charge density and a high polyvalent effect, the so called *"multiplicity",* was furthermore studied in order to solve one of the major problems in effective non-viral cationic gene delivery; a high transfection efficacy with less toxic side effects. The balance between charge density on one side and size/multiplicity on the other has to be focused. Many synthetic polycationic vectors for non-viral gene delivery show high efficiency *in vitro*, but their excessive charge density usually makes them toxic for in vivo applications. The correlation between molecular weight and hydrophobicity thereby plays a major role for low charged polycationic polymers. In contrast to PEI, which can easily condense genetic material via electrostatic interactions, polymers with functional groups might not be sufficient enough to complex DNA. For those polymers a further chain effect depending on the molecular weight might be required.[62] Studies on PAMAM showed that charge density and hydrophobicity by varying the degree of acetylation and benzoylation results in higher transfection efficiencies.[143] Long and coworkers reported on pH-sensitive imidazolium copolymers and investigated their potential synergy of charge density and hydrogen bonding on transfection efficiency. It was evaluated that these water-soluble cationic polyelectrolytes with a tailored charge density and hydroxyl concentration influence the DNA binding, cytotoxicity, and *in vitro* DNA delivery. Introducing hydrogen bonding and electrostatic interactions improves the binding affinity of the polymer and DNA. Lynch and coworkers suggested that the higher amount of hydrogen bonding allows the design of less-toxic polymers due to a reduction in toxic side effects caused by cationic charge density.[144]

Within this thesis, high molecular weight hPG amines should be developed. Besides, the effective charge density on a particular system as well as the balance between the hydroxyl and amine groups as the driving force for a highly efficient gene delivery vector has to be investigated. Too high positive charge density could be toxic against cells; too low loading could not be strong enough to stabilize the formed polyplex. Furthermore, the synergy between multiplicity and effective charge density for *in vivo* siRNA delivery should be addressed.

2 Scientific Goals

This thesis conducted puts forward the detailed evaluation of the polyglycerol amine scaffold. Thereby, the scientific goal was to understand the correlation between size and charge, the influence of the connected polyvalent effect and its size dependency.

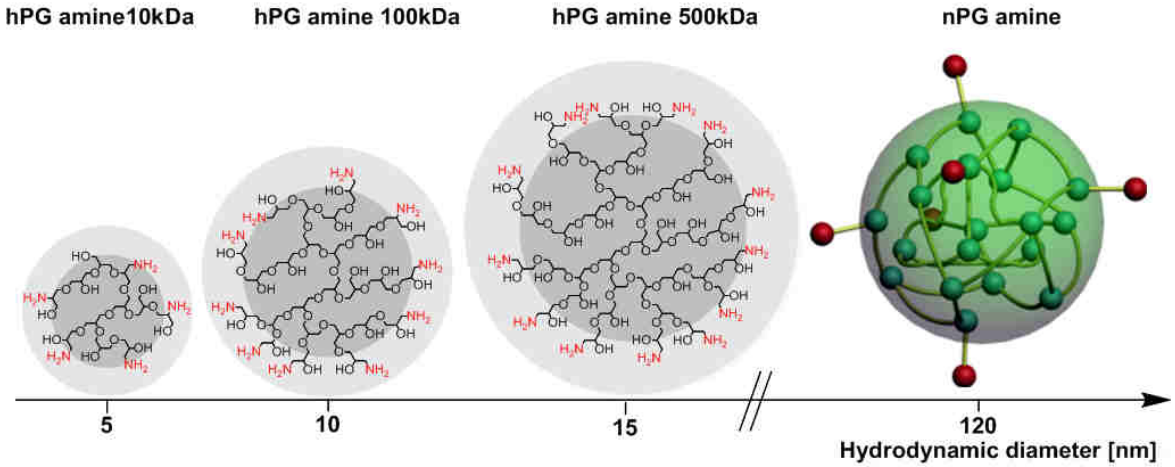


Figure 10. Schematic illustration of the investigated polyglycerol amines of different size and molecular weight. As an example a middle charge density hPG amine is shown.

Several goals should be addressed;

1) Establish a reproducible synthesis for high molecular weight polyglycerols and scale them up.
2) Synthesis of a library of hPG amines varying in size and charge density.
3) Evaluate this set of hPG amines in biological applications concerning size-, charge density- and polyvalent effects.

Taking the expertise of the group in synthesizing hyperbranched polyglycerol (hPG) with the method of the ring opening multi branching polymerization (ROMBP), new conditions should be investigated for a reproducible synthesis of hPG having molecular weights higher than 50 kDa. As a first goal, the synthesis of hPG with a molecular weight of 100 kDa and 500 kDa was successfully addressed, according to the method by D. Brooks *et al.*[113] The dependency of the synthesis on the solvent was thereby described in detail. In consent with our own results two polymeric strategies should be established. The 1,4-dioxane pathway to gain molecular weights of 400-700 kDa or even higher and the tetrahydropyrane (THP) supported synthesis for molecular weights of 50 kDa up to 300 kDa. Finally, the successful scale-up reaction of the hPG 100 kDa should be addressed.

As described before, hPG amine is a basic scaffold without any special functional groups or targeting ligands. But this simple structure is ideal to address the aim to investigate the charge density in correlation to the size in biological applications and to understand the effect of multiplicity. As a first hint, the uptake behavior of two different amine loaded polyglycerol based nanogels (nPG amines) with the same size should be evaluated.

In a second study the in vivo siRNA delivery of an optimized hPG amine with a middle charge density, the 43kDa PG50, should be evaluated. Herein, the limitation on a single molecular weight hPG (8 kDa) with respect to the effective charge density can be demonstrated as well as the optimization of the effective charge density and the resulting knockdown in vivo. The effect based on the multiplicity of the hPG amine with respect to size gives a further hind towards an ideal optimized nanocarrier.

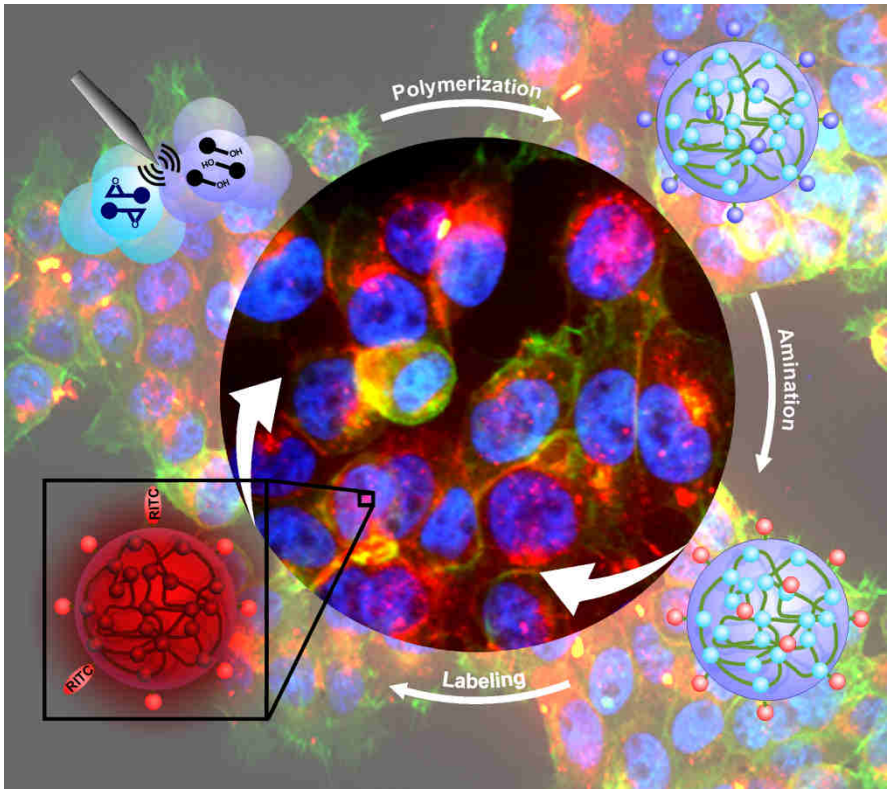
In a third study, different hPG amines should be investigated concerning charge density and size effects on cytotoxicity and DNA delivery. Thereby, the result of the first study should be further evaluated; highly charged hPG amines (>80%) are toxic *in vitro*, which is correlating with the known disadvantages of branched PEI and low charged hPG amines (<15%) are non-toxic. Due to that fact, the study was concentrated on all synthesized moderately charged hPG amines.

3 Manuscripts and Publications

3.1 Functionalized polyglycerol amine nanogels as nanocarriers for DNA

This chapter was published in *Macromol. Biosci. 2014, 14, 1215–1221* http://dx.doi.org/10.1002/mabi.201400144.

Markus Hellmund, Haixia Zhou, Olga Samsonova, Pia Welker, Thomas Kissel and Rainer Haag*



The author's contribution

- Design of the project together with Dr. Haixia Zhou
- Synthesis and characterization of the both high functionalized nPG amines (78% and 88%)
- Ethidium-Bromide, DLS and zeta-potential measurements of the nPG amines in cooperation with Dr. Haixia Zhou
- Establishment and performance of the cytotoxicity studies under supervision of Dr. Olga Samsonova
- Design of the cellular uptake experiment with the help of PD Dr. Pia Welker, sample preparation for the cellular uptake experiments
- Discussion and evaluation of all the results
- Manuscript preparation and submission

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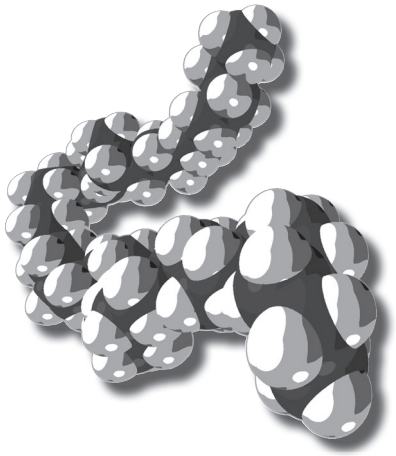

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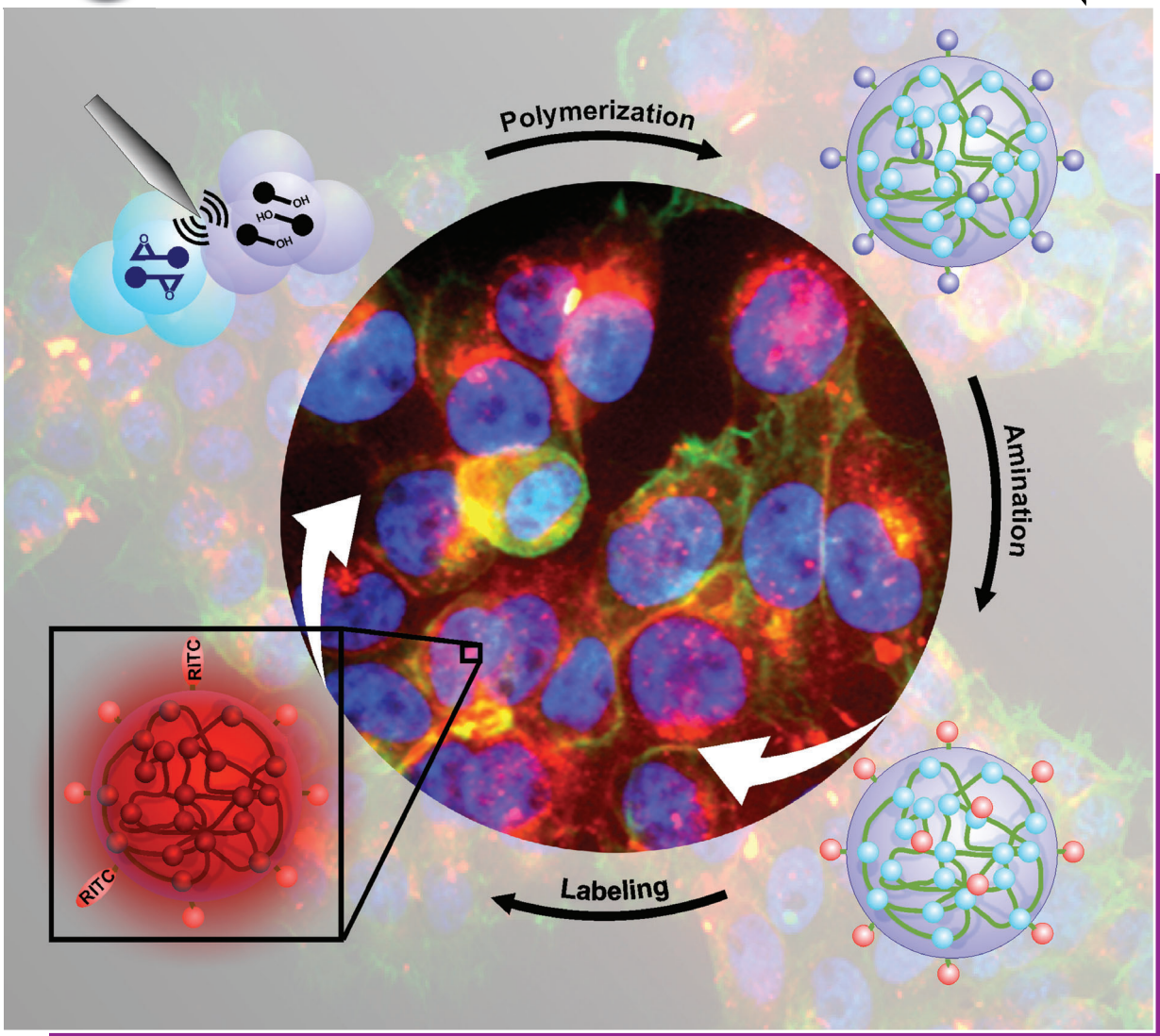

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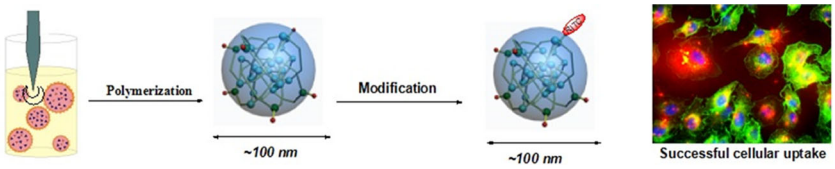
Functionalized Polyglycerol Amine Nanogels as Nanocarriers for DNA[a]

Markus Hellmund, Haixia Zhou, Olga Samsonova, Pia Welker, Thomas Kissel, Rainer Haag*

Polyglycerol based nanogels (nPG) can function as cellular delivery systems. These nPGs are synthesized with different amine densities (nPG amines) by acid-catalyzed epoxide-opening polymerization using a mini-emulsion approach and surface modification. All the synthesized nanogels are characterized by NMR, dynamic light scattering, and ζ-potential, showing slightly positive surface charge and a homogeneous size of ≈100 nm. The use of these systems for delivery applications is demonstrated with regard to polyplex formation, cytotoxicity, and cellular uptake studies. It is depicted that the CE_{50} value of the high loaded nPG amines is eight times higher than the low loaded ones. The influence of the amine loading percentage on the nanogel and the effects of polyvalency in these architecture is discussed.



1. Introduction

In the field of gene delivery systems, non-viral vectors have gained considerable significance[1] as an alternative to viral vectors which are efficient in terms of transfection but suffer from immunogenicity, low loading capacity, and difficulties in up-scaling.[2] For the development of non-viral gene delivery vectors, cationic lipids[3] and cationic polymeric systems[4] have attracted much interest in the literature. Several parameters that influence the efficiency of these cationic systems, such as head groups, targeting ligands, molecular architecture, have been investigated. Cationic polymers offer the added advantage of the "proton sponge effect," which is the most generally accepted mechanism and is believed to be one reason for the efficient transfection of cationic polyplexes.[5] Polyethylene imine (PEI), polypropylene imine (PPI), and polyamido amine (PAMAM) have been reported to have a high DNA/RNA transfection efficiency based on the "proton sponge effect".[4]

In the case of macromolecular systems, several architectures, linear and branched polymers,[4] nano- and microgels[6] as well as dendritic polymer structures[7] have been studied for gene delivery. Branched polymers that are accessible in one-step reactions, in contrast to the multi-step growth of perfect dendritic structures, have also been investigated.[8] The requirements for these branched polymers vary according to their size, charge, and buffer capacity and their hydrophobic and/or hydrophilic character. Hyperbranched aliphatic polyethers architectures are especially suitable for biomedical applications due to their excellent biocompatibility and their multifunctional and well-defined structures. Among them, hyperbranched polyglycerol (hPG) is a promising candidate due to its globular structure with a high density of functionalizable hydroxyl groups and its excellent biocompatibility and biological inertness in vivo.[9] The hPG backbone can easily be synthesized by one-pot anionic ring-opening polymerization and its derivates are attractive for drug and gene delivery.[10][11] However, particles of less than ca. 5 nm rapidly leave the blood stream by extravasation and renal clearance. In contrast, larger nanoparticles with a size of hundreds of nm circulate in the blood stream for a longer time and tend to accumulate in

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[a]**Supporting Information** is available from the Wiley Online Library or from the author.

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leaky tissues (tumor, inflammations) due to the "enhanced permeation and retention effect" (EPR).[12]

Over the last few years, we have developed polyglycerol based nanogels (nPG) as promising candidates for cellular delivery due to their size, high stability, biocompatibility, and controlled responsiveness of external triggers like ion strength, pH-value, and temperature, synthesized by both mini emulsion technique and nanoprecipitation.[12] These nanogels are a special category of high molecular weight polymers with characteristics of defined nanoparticles and cross-linked hydrogels. They combine the features of a tunable size of 20–200 nm and a dense surface functionality like hPG. Additionally, nPG show a high Young's modulus which is a sign of a rigid core structure, with the result that they have been much studied for their stiffness and size.[13] In the search for new polymeric architectures that have a longer circulation half life in blood, the synthesis of different nPGs and studies of their physicochemical properties have been reported.[14] In this paper, we synthesize polyglycerol amine nanogels and study the relation of charge density, their behavior during polyplex formation with DNA and cellular uptake. Furthermore we investigated the polyvalent behavior of these polyglycerol amine nanogels and their physicochemical properties. The polymer network of the nanogels allowed a comparison to the low molecular weight hPG during the cytotoxicity studies, which was performed with one of the high density nPG amines. The influence of the degree of functionalization on the nPG amines was tested via cellular uptake using two different amine functionalizations (16% and 80%).

2. Experimental Section

2.1. Analytical Measurements

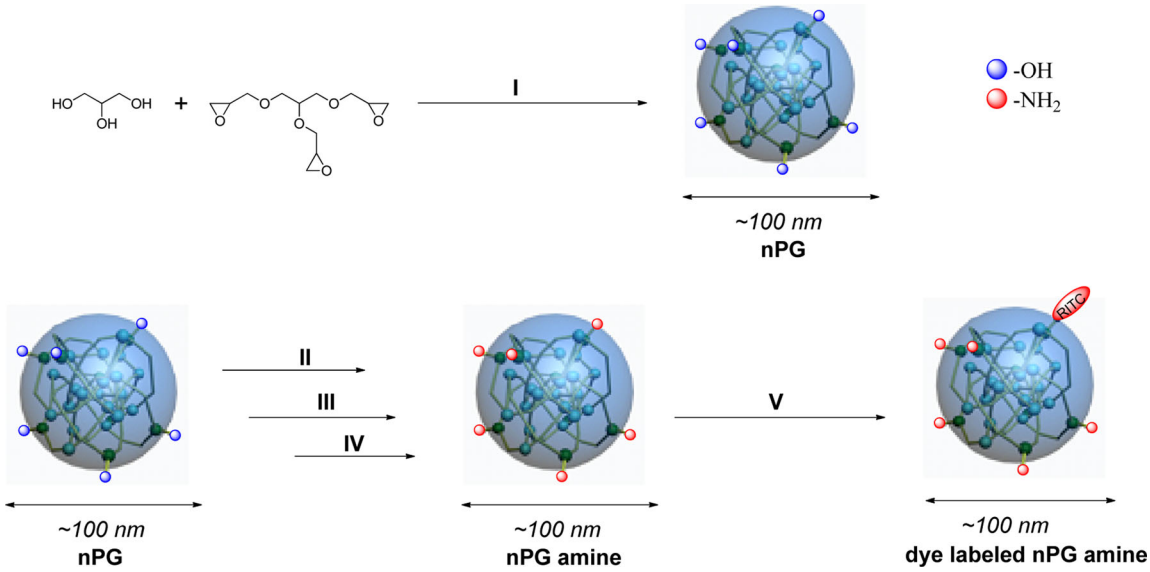
NMR spectra were obtained with the following spectrometers: Bruker ECX 400 (400 MHz proton-resonance) and Bruker AVANCE 700 (700 MHz proton-resonance) (solvent standards, sample amount: ^{1}H-NMR: 10–20 mg, ^{13}C-NMR: 60–120 mg). Solvent calibration was performed according to the literature.[15] Dialysis was performed in benzoylated cellulose dialysis tubes from Sigma-Aldrich (No. D-7884, width: 32 nm, molecular weight cut-off ((MWCO) 1000 g mol^{-1}). 1 L of HEPES-Buffer (2×10^{-3} M HEPES, HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10^{-11} M ethylenediamine tetraacetate (EDTA), 9.4×10^{-3} M sodium chloride) was adjusted to a pH-value of 7.2.

2.2. General Synthesis of nPG Amines and Labeled nPG Amines

All starting materials were commercially available. The absolute solvents were used without further purification. The employed glassware was dried at 700 K under high vacuum (HV) and flushed with argon, respectively, three times just before the reaction. Addition of chemicals was carried out under inert gas conditions.

The nPG were synthesized according to a published procedure. All samples are based on an $A_3 + B_3$ System, glycerol + glycerol-trisglycidylether (GTGE). The modification of hydroxyl groups on the nPGs to primary amines was performed similar to a published procedure for hyperbranched polyglycerol derivates,[16] according to the reaction scheme in Scheme 1.

nPG Synthesis **(1)**: The reaction was performed in two 20 mL sealed tubes. In one tube the surfactant KLE 25 [poly(ethylene-*co*-



Scheme 1. Synthesis of nPG and amine/dye functionalization; polymerization of nPG, amine modification of nPGs, and dye labeling of nPGs; [(I) H^+, KLE25; (II) MsCl, pyridine, 0 °C-rt, 16 h; (III) NaN_3, DMF, 4 h (120 °C), 16 h (60 °C); (IV) PPh_3, rt, 24 h, H_2O/THF 1:1; (V) DMSO, RITC, 90 °C, 2 h].

butylene)-*block*-poly(ethylene oxide)] (20 mg, 2.5×10^{-12} mol) was dissolved in 15 mL of cyclohexane, in the other tube the monomers glycerol **(A)** and GTGE **(B)**, with a molecular weight ratio 3:2 (scale 1 g), were dissolved in dimethylsulfoxide (DMSO). Both tubes were stirred with 1500 rpm for 5 min, then mixed in a 30 mL sealed tube, and stirred for another 1 h at 1500 rpm. Finally the emulsion that formed was sonicated for 1 min under water cooling with an ultrasonic tip apparatus, which was repeated at least four times to obtain a homogeneous mini emulsion. Then a catalytic amount of p-toluenesulfonic acid (P-TSA) was added and the reaction mixture was heated at 393 K for 16 h. Afterwards the reaction was quenched with water and, after cooling down to room temperature, the nanogel was precipitated in n-hexane. The raw product was separated by solid/liquid extraction and finally dialyzed in methanol. Yield: 20–30%. ^{1}H-NMR (700 MHz, CD_3OD, δ): 3.4–4.2 (m, PG-backbone), ^{13}C-NMR (176 MHz, CD_3OD, δ): 62.6– 81.5 (PG-backbone).

O-Mesylpolyglycerol based nanogel **(2):** This reaction was carried out under an inert gas atmosphere. In a two-necked 100 mL flask equipped with a dropping funnel, thermometer, and magnetic stirrer nPG **1** (1 g, 13.5 mmol OH groups) was dissolved in abs. pyridine (15 mL). The solution was cooled down to 273 K using an ice/sodium chloride bath. Finally a solution of methanesulfonylchloride (1 mL, 1.54 g, 13.5 mmol) in abs. pyridine (1 mL) was added drop-wise to the solution, taking care that the reaction temperature did not exceed 278 K. The brown mixture was stirred for 16 h in a thawing ice bath and quenched by adding ice. The solvent was removed under reduced pressure, the residue was dissolved and dialyzed in acetone to give a brown highly viscous product. Conversion: quant., yield: 85%; ^{1}H-NMR (400 MHz, $(CD_3)_2CO$), δ): 5.16 – 4.74 (functionalized secondary OH-groups, nPG), 4.63 – 4.20 (functionalized primary OH-groups, nPG), 3.17 (Me); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$), δ): 83.1 – 69.0 (PG-backbone), 38.2 (Me); IR (KBr): $\nu = 3030$ (w), 2941 (m), 1709 (w), 1457 (w), 1362 (w), 1184 (m), 971 (ν_s), 813 (m), 753 (m) cm^{-1}.

Polyglycerolazide based nanogel **(3):** *O*-mesylpolyglycerol based nanogel **2** (1 g, 13.5 mmol OMs-groups) in a 100 mL one-necked flask with reflux condenser and magnetic stirrer was dissolved in abs. *N, N*-dimethylformamide DMF (15 mL). After addition of NaN_3 (4.39 g, 5 equiv.), the resulting suspension was heated to 393 K for 4 h, finally stirred for 16 h at 333 K behind a transparent security wall. After cooling, the solvent was removed under reduced pressure and the residue was dissolved and dialyzed in chloroform to remove traces of DMF. Conversion: quant., yield: 86%; ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 4.23–2.87 (PG-backbone); ^{13}C-NMR (75 MHz, $CDCl_3$, δ): 81.9–67.5 (PG-backbone), 60.5 (functionalized secondary PG-groups), 51.5 (functionalized primary PG-groups); IR (KBr): $\nu = 2873$ (w), 2102 (s, ν (N_3)), 1457 (w), 1273 (m), 1122 (m), 668 (m) cm^{-1}.

Polyglycerol based nanogel amine (nPG amine) **(4):** Polyglycerolazide based nanogel **3** (0.5 g, 6.75 mmol) was dissolved in tetrahydrofuran (THF; p.a., 15 mL) in a 100 mL one-necked flask. PPh_3 (1.77 g, 6.75 mmol, 1 equiv.) and 5 mL H_2O were added to the solution and N_2 formation was observed. H_2O (10 mL) was added drop-wise over 24 h via dropping funnel to avoid precipitation of the partially reduced product. Finally the solvent was removed under reduced pressure. The residue was dissolved and dialyzed in methanol to give a honey-like product. Conversion: quant.; yield: 85%; ^{1}H-NMR (400 MHz, CD_3OD, δ): 4.2–3.5 (PG-backbone), 3.4 – 3.2 (functionalized PG-groups); ^{13}C-NMR (75 MHz, CD_3OD, δ): 83.0–65.5 (PG-backbone), 55.5–43.6 (functionalized PG-groups); IR (KBr): $\nu = 3354$ (w), 2874 (m), 1576 (s), 1473 (m), 1338 (m), 1104 (m), 820 (m), 668 (m) cm^{-1}.

Synthesis of dye labeled nPG amines **(5):** Rhodamine B isothiocyanate (RITC) labeled nPG amines were prepared following the literature procedure:[17] nPG amine (10 mg) and RITC (1 mg) were dissolved in dry DMSO (1 mL). The reaction mixture was heated at 363 K for 2 h. The solvent was removed under reduced pressure. The residue was dissolved in H_2O. The product was purified from free hydrolysed RITC using a G 25 super fine sephadex column (solvent: H_2O; yield: 80%).

2.3. Ethidium-Bromide Exclusion Assay

The ethidium-bromide (EtBr) exclusion assay was performed as reported in the literature.[18] Fluorescence measurements were obtained on JASCO FP-6500 spectrofluorometer. All samples were excited at 546 nm and had an emission between 560 and 700 nm. A 1.26×10^{-6} M EtBr solution was prepared by dissolving 0.124 mg in 50 mL HEPES Buffer. The DNA single strands (Operon Biotechnologies GmbH, sequence 5′-GAUUAUGUCCGGUUAUGUAUU-3′) were freshly hybridized using the following procedure: The same amount of DNA sense and antisense single strand was weighted and dissolved in HEPES buffer solution. Afterwards, the solution was heated at 363 K for 1 min for hybridization, and then incubated for 60 min in a shaking incubator (310 K, 1200 rpm).

Initially, the fluorescence of pure solution of EtBr and DNA was measured. Then different mixtures were prepared and incubated at room temperature for 30 min to ensure interaction between DNA and EtBr. The maximum fluorescence difference between pure EtBr and EtBr/DNA solution was set to 100%. After adding this constant EtBr/DNA solution to each flask, a different amount of nPG amine solution was added and then filled up with HEPES buffer to 5 mL. These mixtures were incubated 30 min at room temperature before measurement. A pure EtBr solution was measured and set to zero as a negative control.

2.4. Dynamic Light Scattering (DLS) and Zeta (ζ)-potential

DLS measurements were conducted using Malvern Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany) at a constant scattering angle of 173°. All the samples were measured at 298 K and freshly prepared at least 6 h before measurement. All the measurements were repeated at least three times. All the samples were prepared in millipore water (pH value 6.5–7.5) for the hydrodynamic size and for ζ-potential measurements. The polyplexes were prepared at the desired N/P ratio by adding a DNA solution (0.2×10^{-3} M, HEPES-buffer, 21-base pair) and the correlated amount of nPG amine. The polyplex were incubated for 30 min at room temperature and then diluted to 0.7 mL for the measurements.

2.5. Cytotoxicity Studies

Cytotoxicity studies were performed with L929 cell line as described previously.[19] Before testing the cells were seeded in a

density of 8000 cells per well (96-well micro titer plates, Nunclon, Nunc, Germany). The concentration of the samples was chosen to reach or exceed the concentration of 50% inhibition (IC_{50}), starting from 100% blank untreated cells and going down to the survival rate of 10–20%. Briefly, after 24 h of incubation an excess of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was added to every fresh and medium free sample and kept for another 4 h. Afterwards the excess medium was removed and the residual cells with formazan crystals were dissolved in DMSO. The color intensity was measured and detected at 580 nm and corrected with 690 nm background with a plate reader (Titertek Plus-MS 212, ICN, Germany). These results were plotted against the sample concentrations to obtain the IC_{50} value, depicted as mean of triplicates of the tested samples.

2.6. Cellular Uptake Study

The cellular uptake studies were performed by Pia Welker from mivenion GmbH (Berlin, Germany). All samples were labeled with RITC. The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI) and the cytoskeleton with phalloidin 488. The studies were performed on human lung adeno carcinoma epithelial cell line (A549) and human epidermoide carcinoma (A431). The cellular uptake pictures were monitored after 1 h and 4 h. Image acquisition of cellular fluorescence was performed using a confocal microscope LSM 5 Exiter (Zeiss) equipped with a laser diode (excitation at 405 – 425 nm), an Ar^+ laser (excitation at 456, 488, and 514 nm), and a He-Ne laser (excitation at 633 nm). All images were taken at the same exposure time of 200 ms to enable the comparison of the measured fluorescence signals.

3. Results and Discussion

3.1. Synthesis of Amino Functional Nanogels

We have synthesized a series of defined nanogels in a swollen diameter size of ca. 100 nm. These nanogels were obtained by acid-catalyzed epoxide-opening polymerization of glycidol in mini emulsion. The properties of these nanogels, that is, size, degree of branching, and viscosity were studied and could be controlled by varying the functionalities of the monomers and cross linkers. It was shown that the choice of the monomer and the cross linker played a vital role in obtaining the required degrees of branching (DB), sizes, and viscosity.[20] Altogether three nPGs were synthesized. Two of three were modified to get a high density of primary amines, 78% and 88% (targeting 80%, ca. 10.8 mmol g^{-1} amine) and to obtain one with a lower loading 16% (targeting 15%, ca. 2.0 mmol g^{-1} amine) by depicted a three-step synthesis (Scheme 1).

3.2. Polyplex Characterization and DNA Condensation

Since the size and charge of a nanoparticle influences the complexation behavior, all nPG amines were characterized by DLS and ζ-potential (nPG amine 88%: 96.5 ± 1.7 nm, 0.8 ± 3.8 mV; nPG amine 78%: 134.3 ± 17.7 nm, 6.58 ± 2.8 mV; nPG amine 16%: 97.5 ± 0.6 nm, 0.0 ± 4.1 mV). The results demonstrate a homogenous hydrodynamic size of ca. 100 nm for each nPG amine and a similar ζ-potential. All the nanogels were investigated further for their ability to complex DNA and stabilize the formed polyplexes.

The ability of all nanogels to complex DNA was studied at different N/P ratios. The polyplexes were prepared at the desired N/P ratios by adding a DNA solution to the corresponding amount of nPG amine. The results of the ζ-potential measurements and hydrodynamic diameter for the nPG amine polyplexes are summarized in Figure 1. The hydrodynamic size of all polyplexes was in the range 0.2–0.4 μm due to agglomeration during the polyplex formation. It may be that the DNA interacted with more than one nPG amine to build a stable polyplex via electrostatic interactions. Agglomeration of polyamines has been reported in the literature.[21] It is generally accepted that a nearly neutral or low cationic surface charge density will improve circulation half-life by reducing non-specific interactions.[1] As expected, the charge on the surface of both types of compounds caused a totally different complexation behavior. In the case of nPG amine 16%, the low ζ-potential of ca. 2 mV

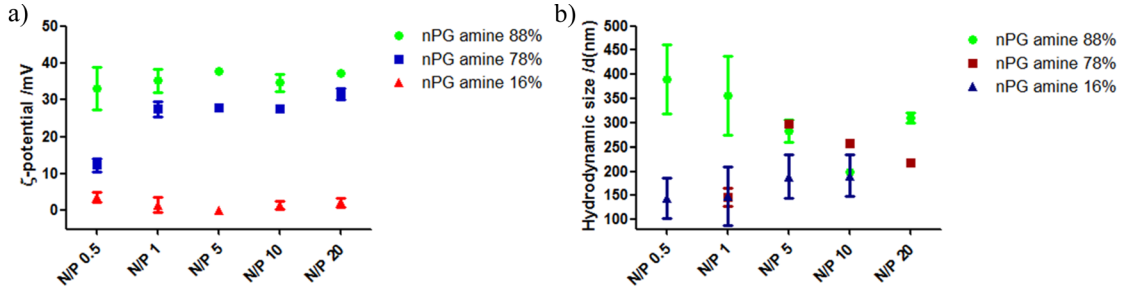


Figure 1. Results on nPG amine polyplexes a) ζ-potential and b) hydrodynamic size. Due to agglomeration at higher N/P ratios, the size measurements of nPG amine 16% are missing.

hinted towards the ideal density of amines for polyplex formation. nPG amine 16% showed a nearly neutral polyplex. The high density nPG amines had a five times higher concentration of amines than the low density one. In the case of the high surface density nPG amines the high ζ-potential of ca. 30 mV led to an excess of positive charge on the surface, forming a stable polyplex due to the strong binding affinity.

To confirm the affinity of the nanogels towards their binding strength to DNA, an additional EtBr exclusion assay was performed. During this assay, EtBr was intercalated into the DNA helical structure. In the presence of another ligand, EtBr could be competitively displaced, followed by the decrease of the fluorescence signal. nPG amine 88% and nPG amine 16% were tested at different concentrations to get the C_{50} values, the loss of 50% of the original fluorescence intensity. This value is inversely proportional to the binding affinity of the tested nanogel. Small values demonstrate a higher DNA binding affinity.[21] Natural oligoamines like spermine, spermidine, etc., are able to condense DNA at CE_{50} values of ca. 10. We have shown that polyglycerol based oligoamines were able to condense DNA at lower CE_{50} values using a low molecular weight hyperbranched polyglycerol as the core with different amine densities.[11] To compare our results with those reported in the literature, the total amount is shown in correlation to the N/P ratio, the so-called CE_{50} value. This value is proportional to the density of amines used in the polyplex formation. The measured CE_{50} values are summarized in Table 1.

The results demonstrate that the high density nPG amine has a stronger binding affinity to the DNA than the low density one as well as hPG based polyamines. nPG amine 16% is similar to spermine, a natural tetra-amine. It is remarkable that the DNA binding affinity of the high density nPG amine is about eight times higher than that of nPG amine 16%, which indicates that the polyvalent effect plays an important role.[22] Due to the reduced entropic effect and sterical stability based on the size of the nanogel, the shielding of DNA is favored over the intercalation of EtBr into the DNA helical structure.[23]

Table 1. Comparison of the CE_{50} values of nPG amines with different oligo- and polyamines.

Sample	CE_{50} value
nPG amine 88%	0.5
hPG based oligoamine[a]	0.6–1.6
nPG amine 16%	4.1
Spermine[a]	5.8
Spermidine[a]	15.0

[a]Data from ref. [11].

3.3. Cytotoxicity and Cell Penetrating Studies

The major disadvantage of most polycationic systems for in vivo applications is their cytotoxicity. We have reported that polyglycerol based polycations showed a significantly lower cytotoxicity than comparable bPEI structures.[7] We could demonstrate that having the same size and different densities of amines influences the cytotoxicity in vitro and in vivo.[11] In order to further investigate whether these new nanogels can act as nanocarrier systems for non-viral gene delivery, we performed two independent in vitro studies.

The investigation of cytotoxicity for the polyglycerol based nanogels was performed using an MTT assay. The resulting IC_{50} values indicated that the toxicity of nPG amine 78% was similar to the hPG amine 80%, although the IC_{50} value of bPEI 25 kDa was below the one reported in the literature (30 $\mu g\,mL^{-1}$ [11]).

The cellular uptake of these nPGs was studied with nPG amine 78% and nPG amine 16%. Both nanogels were fluorescently labeled with the dye RITC as reported in the experimental section. This dye is commonly used during cellular uptake studies as an unspecific fluorescent marker with an absorption maximum of approximately 550 nm. The positioning of the labeled nPG amines makes it possible to follow the cellular uptake. The studies were performed with two different cell lines (A549: human lung adeno carcinoma epithelial cell line and A431: human epidermoid carcinoma cell line). Combination of two different cell lines and their characteristics was necessary to improve the outcome of the cellular uptake studies. Cell line A431 is known to have a high rate of endocytosis, comparable to cell line A549 due to the source area. Cell line A431 has a high regeneration ability which is needed in the epidermis and A549 has the long-living ability of a lung epithelial cell. As described in the experimental section, the cellular uptake of these nanogels was studied using a confocal microscope. Therefore the nucleus was stained additionally with DAPI (blue) and the cytoskeleton with phalloidin 488 (green). These stainings allowed the characterization of the cells during cell uptake which is shown after 4 h in Figure 2. One additional measurement was performed after 1 h, Supporting Information). Both labeled nanogels were dissolved in phosphate buffer solution (PBS) and tested under physiological conditions. The experiments demonstrated successful uptake of the nPG amines by both cell lines.

nPG amine 16%-RITC showed an unique distribution after 4 h as well as nPG amine 78%-RITC. However, nPG amine 78%-RITC seems to have agglomerated during the 4 h incubation time, which might be due to the lower solubility of nPG amine 78%-RITC in PBS buffer and the higher amine

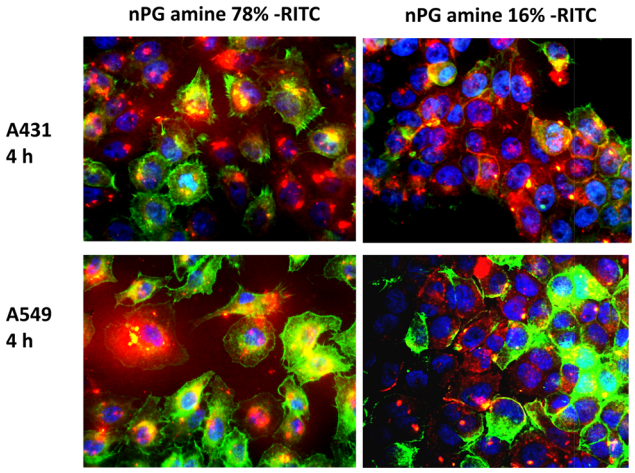


Figure 2. Cellular uptake pictures measured after 4 h with both dye labeled nPG amines. The nuclei are stained with DAPI (blue); nPG amines with RITC (red), cytoskeleton with Phalloidin 488 (green), A549: human lung adeno carcinoma epithelial and A431: human epidermoide carcinoma cell line.

density than with nPG amine 16%-RITC. The ability to agglomerate was already observed in the hydrodynamic size measurements (see above). All these studies inferred that a nearly neutral nPG with low density of amines can be taken up into cells and seems to be better than the one with high density of amines.

4. Conclusion

We have synthesized a new series of polyglycerol amine nanogels as potential non-viral gene delivery systems, and demonstrated their ability to complex DNA forming stable polyplexes. The results from ζ-potential and EtBr assay showed that high density nPG amines led to eight times higher binding affinity towards DNA, as compared to the lower density ones and different hPG oligoamines reported previously. The almost neutral polyplex formed by the low density nPG amine was still able to remove the intercalated EtBr to build a more stable polyplex compared to natural oligoamines. We could show that the physicochemical properties and a successful cellular uptake depend on the polyvalency of the amine groups. Furthermore, our nanogel architecture showed a lower cytotoxicity compared to the bPEI standard. All these results clearly indicate that nPG amine architectures are good candidates for DNA complexation and cellular uptake.

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Supporting Information

for *Macromol. Biosci.*, DOI: 10.1002/mabi.2013#####

Functionalized polyglycerol amine nanogels as nanocarriers forDNA

Markus Hellmund, Haixia Zhou, Olga Samsonova, Pia Welker, Thomas Kissel and Rainer Haag*

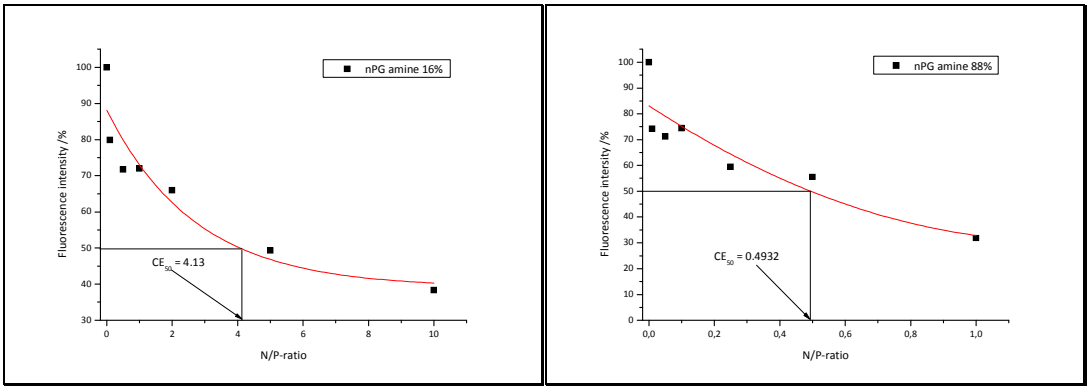


Figure S 1 CE_{50} value of the tested nPG amine polyplexes (Fluorescence intensity (%) vs. N/P ratio).

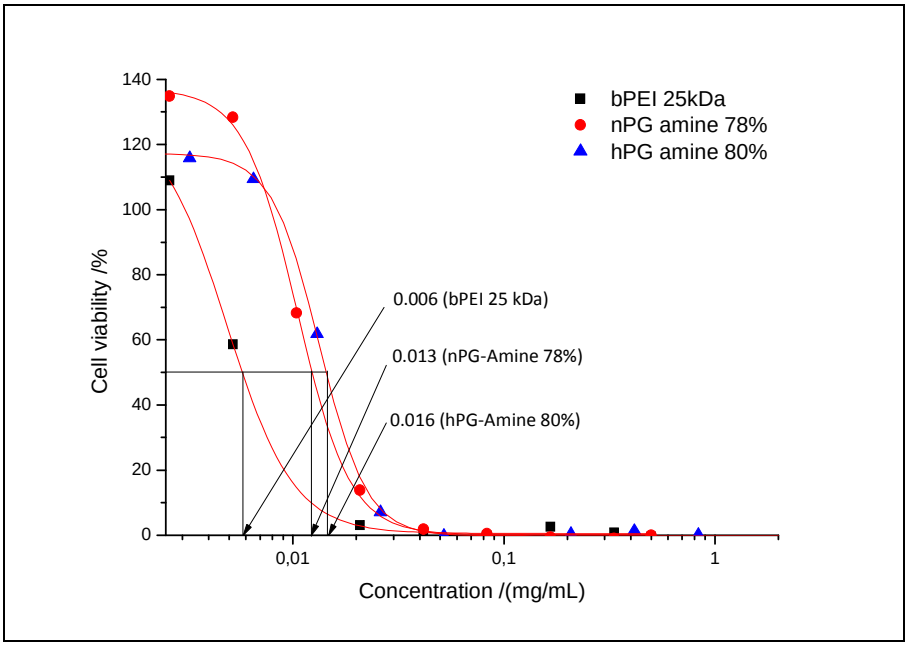


Figure S 2. IC_{50} values of the tested compounds shown as cell viability (%) vs. concentration (mg mL^{-1}).

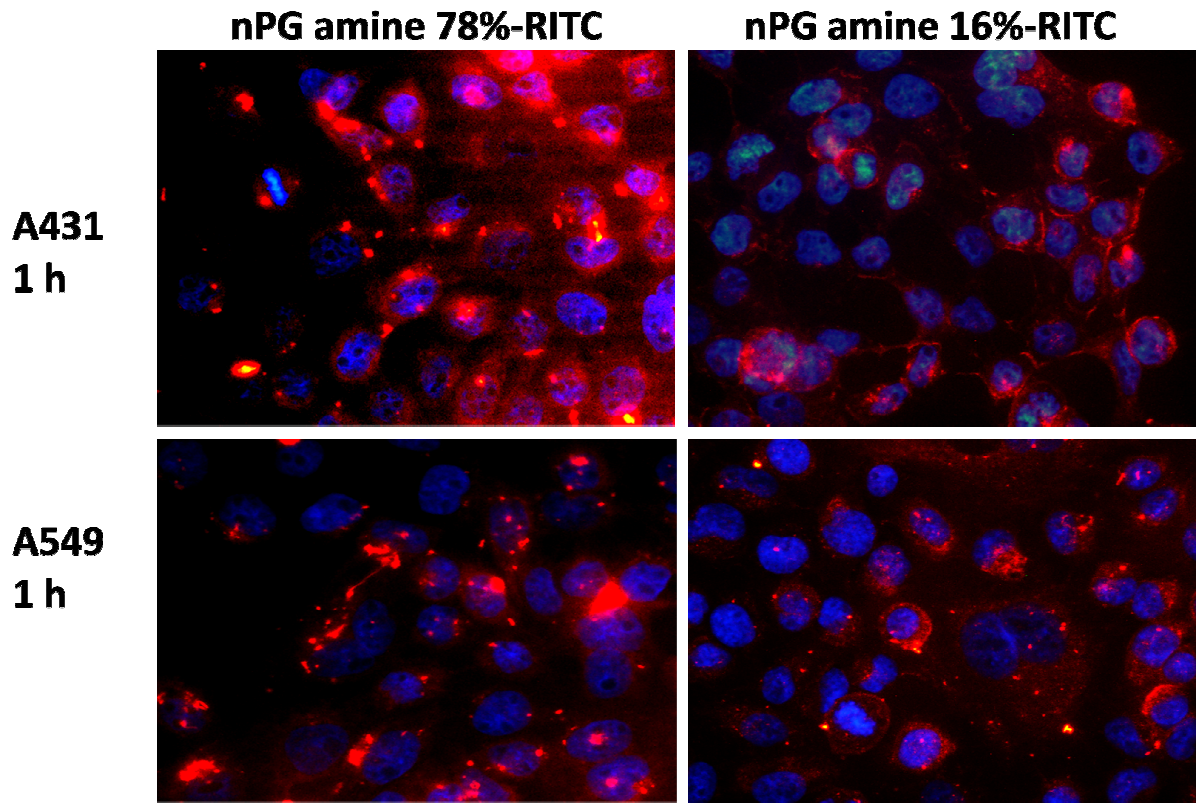


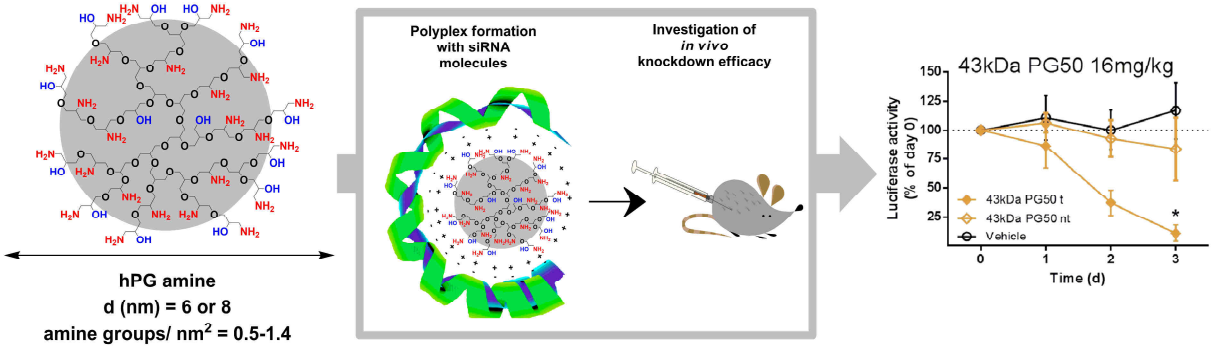
Figure S 3. Cellular uptake pictures measured after 1 h. Nucleus was stained with DAPI (blue). nPG amines stained with RITC (red). nPG amine 16% showed cellular uptake in a homogenous distribution. nPG amine 78% displayed agglomeration spots and cellular uptake. Both compounds were taken up in the tested cell lines, A549: human lung adeno carcinoma epithelial and A 431: human epidermoid carcinoma cell line.

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

This chapter is submitted to Journal of Controlled Release

Anna Maria Staedtler,[†,a] Markus Hellmund,[†,b] Fatemeh Sheikhi Mehrabadi,[b] Bala N. S. Thota,[b] Thomas M. Zollner,[a] Markus Koch,[a] Rainer Haag[b] and Nicole Schmidt[a,*]

[†]*Both authors are contributing equally*



The author's contribution

- Design of the project together with Anna Maria Städtler und Dr. Nicole Schmidt
- Synthesis and characterization of both 43 kDa hPG amines
- DLS and zeta-potential measurements of the 43 kDa hPG amines
- Establishment and performance of the gel electrophoresis evaluation for all hPG amines
- Calculation of the amine density per surface area for all hPG amines
- Discussion and evaluation of all chemical results
- Discussion of all the biological results and manuscript preparation + submission together with Anna Maria Städtler
- Design of the Graphical Abstract Figure together with Anna Maria Städtler

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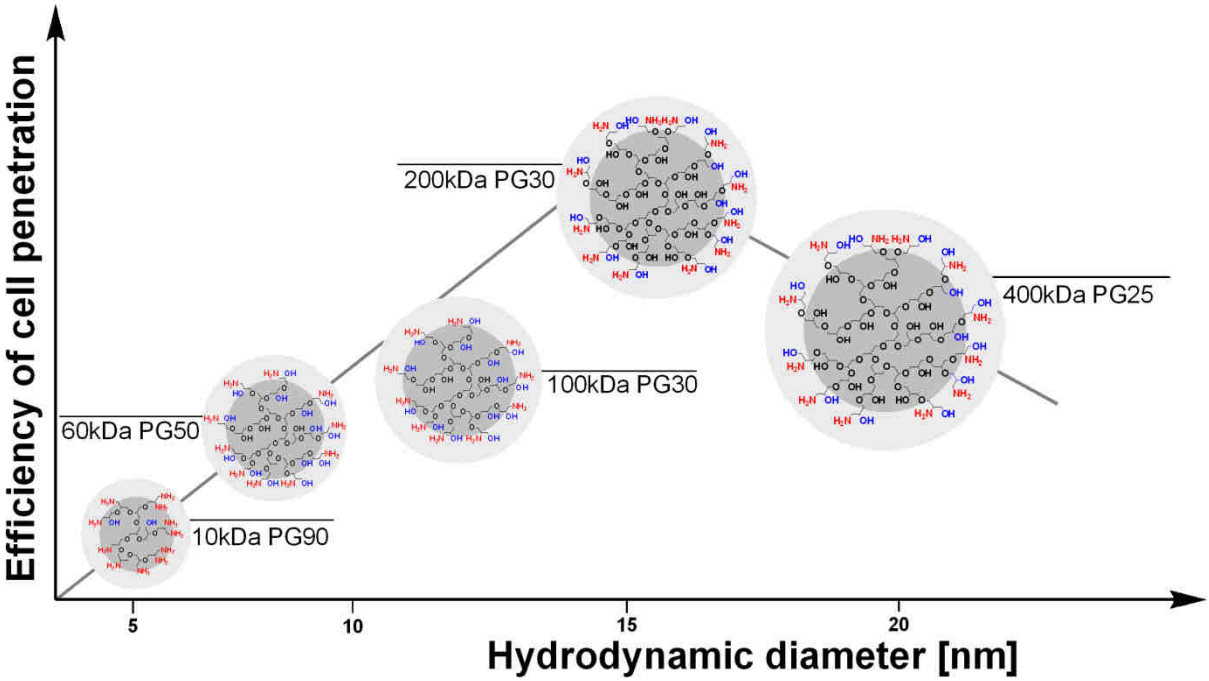
3.3 Systematic adjustment of charge densities and size of hPG amines reduces cytotoxic effects and enhances cellular uptake

This chapter is submitted to *Biomaterial Science,*

http://dx.doi.org/10.1039/C5BM00187K - Reproduced by permission of The Royal Society of Chemistry

Markus Hellmund,[a] Katharina Achazi,[a] Falko Neumann,[a] Bala N. S. Thota,[a] Nan Ma,[a,b,*] and Rainer Haag[a,*]



The author's contribution

- Design of the project
- Synthesis and characterization of all hPG amines
- Chemical evaluation of all hPG amines
- Design the strategy of the biological evaluation together with Dr. Katharina Achazi
- Manuscript preparation from the chemical side and final approval/submission

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4 Summary and Outlook

The scientific goals of this work were the synthesis of a library of various high molecular weight hPG amines and their evaluation in biomedical applications *in vitro* and *in vivo*.

As a first step, the synthetic pathway for high molecular polyglycerol was investigated. Thereby, the dependency of the resulting molecular weights on the used solvent was studied. Using 1,4-dioxane led to molecular weights higher than 400 kDa. Changing the solvent to THP enables the synthesis of 50 kDa up to 300 kDa. Furthermore, the ratio between the monomer and the solvent directly correlates with the final molecular weight, for instance, a molecular weight of 100 kDa was reached with an 1:2 ratio of THP:glycidol. All these results were produced in parallel to the research group of D. Brooks and published similarly by them in 2013.[113] The second aim, the up-scaling of the reaction was successfully performed for 100 kDa hPGs, with batch sizes up to 120 g. (Submitted to *Biomaterial Science*) Conclusively, the scientific goal to establish synthetic pathways for higher molecular weight polyglycerols in the research group of Prof. Haag was successfully performed as well as the up-scaling in the case of the 100 kDa hPG.

The biomedical evaluation of different hPG amines as non-viral gene delivery vectors was shown in three different strategies. As a first hint, two different loaded nPG amines were synthesized and their charge dependently cellular uptake was studied. It was shown that the lower charged nanogel exhibits a better cellular uptake, but was less efficient in the ability of binding to DNA. In contrast the higher loaded one had a 10 times better binding affinity, but due to the high charge density, its toxic side effects were enhanced dramatically. In the second study, performed in parallel to the third, the effects of charge density and size were evaluated *in vitro* and *in vivo*. With the restriction of a maximal effective charge density on one hPG core, two different sizes were tested. It could be shown that there is a linear dependency between the multiplicity of amine groups and the zeta potential. Furthermore, the optimized candidate related to size, charge and multiplicity performed the best. It was demonstrated that this moderately loaded nanocarrier can challenge the standard *"invivofectamin"*. These really awesome results are in one line with the ones from the third study. In this one, a library of hPG amines, with varying molecular weight and loading of amine groups, was tested for DNA transport *in vitro*. It was shown that high and low loaded hPG amines are useless for DNA delivery, because they are either too toxic or not strong enough to complex the DNA sufficiently. Only the moderate loaded hPG amines showed really great and sufficient DNA delivery into the cells during cellular uptake. Interestingly, the best candidate had a molecular weight of 200 kDa, which was neither the biggest size nor the best charge density.

Taken together, all scientific goals were successfully addressed. I was able to synthesize hPG amines with loadings from 1-100% and sizes from 50 kDa up to 700 kDa. The size to charge dependency as well as the multiplicity could be shown, resulting in an efficient matrix, which can be used as basis for further studies. With this in hand, we are now able to challenge every special biological application needing an adjustable carrier system.

As an outlook, these scaffolds can be used as carriers for proteins, amino acids or sugar moieties, due to their ability to selectively adjust size and surface properties. Based on electrostatic interactions or covalent bonds, the cellular uptake can be improved and the efficiency of the counterpart can be increased. Furthermore, the hPG amine scaffold can be used with targeting ligands to enhance the ability of selective targeting. Such an optimized system could be able to reach the site of action more efficiently as shown in the second study and improve its efficiency.

5 Zusammenfassung und Ausblick

In der vorliegenden Arbeit wurde eine Vielzahl verschiedener Polyglycerol Amine (hPG Aminen) synthetisiert und evaluiert. Die dabei im Wesentlichen verfolgten wissenschaftlichen Ziele waren zum einen die reproduzierbare Herstellung unterschiedlich großer hPG Amine und zum anderen ihre Verwendung als nicht virale Gentransporter *in vitro* und *in vivo*.

Zu Beginn der Arbeit wurde die Synthese hochmolekularer hPG Amine etabliert. Es zeigte sich, dass allein durch den Wechsel der Lösungsmittel zwei unterschiedlich große Molekulargewichtsbereiche erzielt werden konnten. Unter Verwendung des Lösungsmittels 1,4-Dioxan wurden Molgewichte über 400 kDa erreicht, bei THP zwischen 50 kDa und 300 kDa. Dabei ist das Verhältnis von Glycidol zu THP der entscheidende Faktor für die finale Größe des Partikels, zum Beispiel bei einem 2:1 Verhältnis von Glycidol zu THP erreicht man ein Molekulargewicht von 100 kDa. Die Forschungsgruppe um D. Brooks (Kanada) erzielte zeitgleich ähnliche Ergebnisse über die lösungsmittelabhängige Synthese hyperverzweigter hochmolekularer Polyglycerole und publizierte sie als erste 2013.[113]

Nach erfolgreicher reproduzierbarer Synthese unterschiedlich schwerer hPG Amine wurde in der finalen Phase der Doktorarbeit die großtechnische Produktion von 100 kDa Polyglycerolaminen durchgeführt und Ansatzgrößen bis zu 120 g verwirklicht.

Die biologische Evaluierung der hergestellten hPG Amine als nicht-virale Gentransporter wurde in drei unterschiedlichen Studien durchgeführt. In der ersten wurde die zellulare Aufnahme zweier nPG Amine in Abhängigkeit der Beladungsdichte von Amingruppen an der Oberfläche untersucht. Diese Studie gab einen ersten Einblick in das Verhalten eines extrem positiven und einem fast neutralen Systems. Während das stark positive nPG Amin eine 10-fach höhere DNA Bindungsstärke aufwies, überzeugte das fast neutrale nPG Amin mit einer wesentlich besseren Aufnahme in die Zelle. Ähnliche Ergebnisse wurden in den zwei weiteren Studien beobachtet. Die zweite Studie untersuchte die Abhängigkeit der Transfektionseffizienz von der effektiven Ladungsdichte. Da die Ladungsdichte kerngrößenabhängig ist, wurden zwei Kerngrößen untersucht; ein 8 kDa und ein 43 kDa hPG Amin mit fünf verschiedenen Aminbeladungen. Es stellte sich heraus, dass das 43 kDa hPG Amin mit der besten effektiven Ladungsdichte auch die beste Knockdown Effizienz aufwies, bzw. zu vergleichbaren *in vivo* Ergebnissen wie der Goldstandard „Invivofectamin“ führte und dabei sogar eine bessere Effizienz erreichte. In der parallel zur zweiten durchgeführten dritten Studie wurde eine Vielzahl von unterschiedlich großen und beladenen hPG Aminen untersucht. Dabei zeigte sich, dass alle Partikel mit

Aminbeladungen über 80% *in vitro* zelltoxisch wirkten. Beladungsgrade von unter 15% erreichten keine effektive Stabilisierung der DNA, waren dafür aber komplett nicht toxisch. Deshalb wurde die Studie auf alle moderat geladenen hPG Amine konzentriert. Entgegen der Ergebnisse der zweiten Studie war nicht das 400 kDa hPG Amin mit der besten effektive Ladungsdichte das effizienteste Transportsystem, sondern das hPG Amin mit einer Kerngröße von 200 kDa.

Zusammenfassend ist festzuhalten, dass alle wissenschaftlichen Ziele der Arbeit erfolgreich umgesetzt wurden. Es wurden verschiedene hPG Amine mit Aminbeladungen von 1 bis 100% und mit Molekulargewichten von 50 kDa bis 700 kDa hergestellt. Die größenabhängige und ladungsabhängige Wirkung der Systeme *in vitro* und *in vivo* wurde evaluiert und es konnte gezeigt werden, dass moderat beladene hPG Amine die besseren Transportsysteme sind.

Als Ausblick sollte man auf Basis der gewonnenen Erkenntnisse die weitere Optimierung dieser einfach zugänglichen Transfektions Systeme erwägen. Zum einen könnte die Selektivität gegenüber einer ganz bestimmten Zelllinie weiter entwickelt werden. Dies wird im Allgemeinen durch die Einführung von unterschiedlichen Targetingeinheiten erreicht. Mit Erhöhung der Selektivität könnte man ggf. die Transfektionseffizienz weiter steigern. Zum anderen kann man das Gerüst als Transporter für Peptide, Aminosäuren oder Kohlenhydrate benutzen, um deren Aufnahme in die Zelle zu fördern. Da Peptide eine geringe eigene Aufnahme in die Zelle haben, wäre ein z.B. auf elektrostatische Interaktionen basierter Transport mit den hPG Aminen möglich.

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7 Curriculum Vitae

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