Delivery systems for siRNA: Towards targeted inhibition of tumor angiogenesis

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Delivery systems for siRNA: Towards targeted inhibition of tumor angiogenesis

Afleversystemen voor siRNA:
Op weg naar gerichte remming van tumor angiogenese
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 23 januari 2012 des middags te 12.45 uur

door

Pieter Vader

geboren op 25 april 1985 te Utrecht

Promotoren: Prof.dr. G. Storm
Prof.dr. J.F.J. Engbersen

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“Who has seen the wind?
Neither you nor I.
But when the trees bow down their heads,
The wind is passing by.”

Christina G. Rossetti (1830-1894)

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Chapter 1

General introduction

This chapter is adapted from a manuscript that has been accepted for publication in:
Current Topics in Medicinal Chemistry

TUMOR ANGIOGENESIS

Angiogenesis is the development of new blood vessels out of pre-existing vasculature and is a well-established target in anti-cancer therapy. The primary driving force for tumor angiogenesis is the demand for oxygen and nutrients by growing cancer cells. Angiogenesis is necessary for tumors to expand beyond 1–2 mm^3 in size and is controlled by a balance in pro- and anti-angiogenic factors. Once the radius of the tumor extends beyond the effective diffusion limit for oxygen, this balance can shift in favor of pro-angiogenic factors and the tumor can acquire an angiogenic phenotype, also referred to as the angiogenic switch (1). In response to these factors, endothelial cells are activated, resulting in invasion of the extracellular matrix, migration to the site of recruitment, proliferation and finally vessel formation and stabilization (2). The most critical pro-angiogenic factor appears to be vascular endothelial growth factor (VEGF). VEGF stimulates endothelial cells following binding to integral membrane tyrosine receptor kinases VEGFR-1 and VEGFR-2, which results in activation of multiple intracellular effectors, including the Rho family of small GTPases.

As endothelial cells play a pivotal role in the process of angiogenesis, they are excellent target cell types for anti-angiogenesis-based therapeutic strategies. Presumed advantages of such a treatment strategy as compared to conventional chemotherapy include:

- Limited side effects (as normal vasculature is quiescent)
- Broad applicability (as many tumor types require neovasculature)
- Limited development of resistance (as endothelial cells, unlike tumor cells, are genetically stable)
- Accessibility (as endothelial cells are readily accessible from the blood circulation)

RNA INTERFERENCE

RNA interference (RNAi) is a conserved mechanism for regulation of gene expression in cells. Fire et al. discovered that introduction of double stranded RNA (dsRNA) can lead to silencing of gene expression in *Caenorhabditis elegans* (3). Three years later, this phenomenon was also demonstrated in mammalian cells (4). Since its discovery, RNAi has emerged as one of the most powerful tools in the study of functional genomics and offers hope as a new therapeutic strategy for various diseases, including cancer.

The RNAi response is triggered by dsRNA present inside cells. The dsRNA is recognized by Dicer, a RNAse III-type enzyme, which cleaves it into short fragments of approximately 21-23 nucleotides long, also known as small interfering RNA (siRNA) (5). The siRNA is then incorporated in the RNA-induced silencing complex (RISC), which becomes activated, cleaves the sense strand of the siRNA and is guided by the

antisense strand to degrade mRNA that is recognized via complementary base-pairing (6). The antisense-loaded RISC can then move on to destroy additional mRNAs, which makes RNAi via RISC a powerful catalytic event (7).
siRNA can easily be produced synthetically and then directly introduced into cells. Theoretically, any gene can be silenced when the siRNA is properly designed, which gives application of RNAi with siRNA a very broad therapeutic potential. However, before widespread use of RNAi in a clinical setting is possible, a few hurdles have to be overcome. Due to its relatively large molecular weight (approximately 13 kDa) and highly negative charge, siRNA cannot readily cross cellular membranes. Furthermore, it is susceptible to degradation by endogenous enzymes, like serum nucleases, and rapidly cleared by the kidneys upon systemic administration (8). At this moment, the success of RNAi-based therapy is dependent on delivery systems that modulate the pharmacokinetics and intracellular trafficking of siRNA. These systems should protect siRNA against degradation, reduce distribution to non-target sites and facilitate cellular uptake by the target cells and release within the cytoplasm.

DELIVERY SYSTEMS FOR SIRNA

Various approaches for the delivery of siRNA have been proposed. Generally two classes of delivery systems can be distinguished, namely viral and non-viral carriers. Although viruses possess outstanding transfection efficacies, their inherent drawbacks are limited loading capacity, complicated large scale production and, most meaningful, severe safety risks due to immunogenicity after repeated administration. This gives them only limited potential for broad clinical applications (9). Non-viral delivery systems, which comprise cationic polymers and lipids, may circumvent some of the shortcomings of viruses. They can be specifically tailored for the proposed application and demonstrate adequate safety profiles. However, relatively low transfection efficiency is still a major hurdle that needs to be overcome.

OBJECTIVES

This thesis focuses on the development of non-viral delivery systems for siRNA to inhibit tumor angiogenesis. So far, clinical successes of RNAi-based strategies to inhibit tumor angiogenesis have been limited due to (1) scarcity of clinically promising pharmacological targets and/or (2) inefficiency or toxicity of the siRNA carrier system. In this thesis, we attempted to improve both siRNA target and carrier. Therefore, we studied a novel small Rho GTPase protein, Rac1, as a potential target for anti-angiogenic therapy **(Chapter 2)**. At the same time, we tried to adapt and improve a novel class of biodegradable poly(amido amine)s, with built-in characteristics for intracellular release

of its payload, for gene silencing applications and optimized the carrier to increase its stability in physiological media **(Chapter 3-6)**. Additionally, we tried to modify a previously described lipid-based carrier for siRNA to target angiogenic endothelial cells **(Chapter 7-8).**

OUTLINE OF THE THESIS

In **Chapter 2**, the role of the small Rho GTPase Rac1 in angiogenesis was investigated using RNA interference. The effects of knockdown of Rac1 in endothelial cells on VEGF-induced angiogenesis *in vitro* were explored using tube formation, migration, invasion and proliferation assays. Furthermore, the possibility of using siRNA against Rac1 to inhibit tumor angiogenesis *in vivo* was studied in a Matrigel plug assay and a murine model of neuroblastoma.

Chapter 3 provides an overview of recent advances in the development of polymeric carrier systems for delivery of siRNA. Structural variations that have been applied to these polymers for optimizing their intracellular trafficking are discussed, as well as strategies for stabilization and targeting to diseased tissues *in vivo*.

For biological studies on the efficiency of carrier-mediated uptake of siRNA, quantitative determination of the amount of internalized siRNA is required. In **Chapter 4**, a novel approach for quantifying cellular uptake of fluorescently labeled siRNA is described.

In **Chapter 5**, a new series of biodegradable poly(amido amine)s with disulfide linkages in the backbone was synthesized out of N,N'-cystaminebisacrylamide (CBA), 4-amino-1-butanol (ABOL) and ethylene diamine (EDA). Effects of different percentages of butanolic side chains and protonatable fragments in the main chain on siRNA complexation, cellular uptake, gene silencing and toxicity were investigated.

Chapter 6 reports the synthesis of a PEGylated polymer based on the most successful polymer from Chapter 5. Mixtures of the PEGylated and unPEGylated polymer were used in order to vary the PEG content in the final polyplex. Polyplexes with different PEG content were compared to corresponding unPEGylated complexes regarding physicochemical characteristics, stability, cellular uptake, gene silencing activity and *in vitro* biocompatibility.

In **Chapter 7** an overview is provided of currently employed lipid-based delivery systems for siRNA, and of recent research into improvements of cellular delivery by enhancing target cell uptake and redirecting intracellular trafficking routes. In addition,

the role of lipids in immune activation is discussed, and possible ways to overcome the response are highlighted.

In **Chapter 8**, a targeted Liposome-Polycation-DNA (LPD) siRNA delivery system was employed for inhibition of expression of VEGFR-2, a validated target in angiogenesis, as model protein in angiogenic endothelial cells. RGD-targeted PEGylated LPD particles containing VEGFR-2 siRNA were prepared and optimized for protamine content, DNA content and PEGylation density, and subsequently characterized with regard to their uptake and VEGFR-2 silencing efficacy in murine and human endothelial cells.

Chapter 9 summarizes and discusses the findings and conclusions from this thesis.

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Chapter 2

Examining the role of Rac1 in tumor angiogenesis and growth: A clinically relevant RNAi-mediated approach

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ABSTRACT

Angiogenesis, the sprouting of new blood vessels from the pre-existing vasculature, is a well-established target in anti-cancer therapy. It is thought that the Rho GTPase Rac1 is required during vascular endothelial growth factor (VEGF)-mediated angiogenesis. In the present study, we have used a clinically relevant RNA interference approach to silence Rac1 expression. Human umbilical vein endothelial cells were transiently transfected with non-specific control siRNA (siNS) or Rac1 siRNA (siRac1) using electroporation or Lipofectamine 2000. Functional assays with transfected endothelial cells were performed to determine the effect of Rac1 knockdown on angiogenesis *in vitro*. Silencing of Rac1 inhibited VEGF-mediated tube formation, cell migration, invasion and proliferation. In addition, treatment with Rac1 siRNA inhibited angiogenesis in an *in vivo* Matrigel plug assay. Intratumoral injections of siRac1 almost completely inhibited the growth of grafted Neuro2a tumors and reduced tumor angiogenesis. Together, these data indicate that Rac1 is an important regulator of VEGF-mediated angiogenesis. Knockdown of Rac1 may represent an attractive approach to inhibit tumor angiogenesis and growth.

INTRODUCTION

During angiogenesis, new blood vessels sprout from the pre-existing vasculature. Physiological angiogenesis is important in embryonic development and postnatally, this process plays a role in wound healing and repair, during the female reproductive cycle and placentation during pregnancy. Aberrant angiogenesis can lead to vascular overgrowth which occurs in age related macular degeneration, psoriasis, rheumatoid arthritis and cancer (1). Angiogenesis is necessary for tumors to grow beyond 1-2 mm^3 in size and is controlled by a balance in pro- and anti-angiogenic factors. However, a tumor can acquire an angiogenic phenotype, also referred to as the angiogenic switch, when this balance is shifted in favor of pro-angiogenic factors (2). In response to these factors, endothelial cells are activated, resulting in invasion of the extracellular matrix, migration to the site of recruitment, proliferation and vessel formation and stabilization (3). Arguably, the most critical pro-angiogenic factor is vascular endothelial growth factor (VEGF-A). VEGF stimulates endothelial cells following binding to integral membrane tyrosine receptor kinases VEGFR-1 and VEGFR-2, which results in activation of multiple intracellular effectors, including the Rho family of small GTPases.

Rho GTPases are small molecule members of the Ras superfamily of small GTPases which function as molecular switches in the cell. These proteins broadly play a role in an array of cellular processes such as cell polarity, transcription factor activity, membrane transport, cytoskeleton regulation and vesicle trafficking (4). Rho GTPases cycle between a guanine diphosphate (GDP) bound, inactive state, and a guanine triphosphate (GTP) bound active state. In the active state, Rho GTPases can bind effector proteins and transduce downstream signals from various receptors. It is likely that Rho GTPases play an important role in critical aspects of the angiogenesis process, and therefore represent an attractive target for cancer therapy (5, 6). The most studied Rho GTPases include Rac1, Cdc42 and RhoA, of which Rac1 appears to be the major GTPase responsible for VEGF mediated angiogenesis (7). Rac1 is ubiquitously expressed. It is required at the leading edge of the cell to regulate actin polymerization and to induce membrane ruffling and formation of lamellopodia (4, 8). Rac1 also plays a key role in EC lumen and tubule formation (9, 10). Furthermore, endothelial specific excision of Rac1 leads to defective development of vessels and embryonic lethality, supporting an essential role in vascular development (11). The involvement of Rac1 in tumorigenesis is indicated by several reports demonstrating overexpression or increased activation of Rac1 or its effectors in a variety of cancers (12-14).

In the current study, we have employed RNA interference to selectively silence Rac1 expression. RNA interference is a novel approach in which delivery of small interfering RNAs (siRNAs) into the cytoplasm of target cells induces sequence-specific gene silencing (15) and offers great potential as a therapeutic strategy. We have explored the

effects of Rac1 knockdown on VEGF induced angiogenesis and tumor growth *in vitro* and *in vivo*. Our data implicate Rac1 in the tumor angiogenesis process and as such, Rac1 may represent a potential target for anti-cancer therapy.

MATERIALS & METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza, Verviers, Belgium) were grown in endothelial cell growth medium-2 (EGM-2) (Lonza), consisting of endothelial basal medium-2 (EBM-2) supplemented with a SingleQuots kit (containing growth factors, 2% FBS and antibiotics). Cells were used between passages 3–7. Neuro2A (murine neuroblastoma) cells (ATCC CCL-131) were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B).

siRNAs

siRNAs were purchased from Eurogentec (Maastricht, The Netherlands). Sequence of Rac1 siRNA against the human sequence was 5'-AAG-GAG-AUU-GGU-GCU-GUA-AAA-3' and 5'-UUU-UAC-AGC-ACC-AAU-CUC-CUU-3' and sequence of Rac1 siRNA against the murine sequence was 5'-GAC-GUG-UUC-UUA-AUU-UGC-UTT-3' and 5'-AGC-AAA-UUA-AGA-ACA-CGU-CTT-3'. As control sequence, a negative control siRNA (Eurogentec) (siNS) was used.

Transfection

1.0 x 10^5 HUVECs were resuspended in 500 µl EGM-2 containing 700 pmol of siRNA and transferred into 4mm gap electroporation cuvettes from Harvard Apparatus (Holliston, Mass.). Cells were electroporated for 70ms at 180V, using an ECM 830 Electroporation system (Harvard Apparatus). After electroporation, cells were seeded in a 6-well plate and EGM-2 was added to a total volume of 2 ml per well. After 4 hours, cells were washed 3 times with phosphate buffered saline (PBS) and fresh medium was added. Alternatively, 8.0 x 10^4 cells were seeded in 6-well plates and incubated for 24 hr. Medium was replaced with serum-free medium and cells were transfected with 100 pmol siRNA per well using Lipofectamine 2000(Invitrogen, UK). After 4 hours, medium was replaced with EGM-2.

Western Blot analysis

48 hr following transfection, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and EDTA for 30 min on ice. Following removal of debris by centrifugation, lysates were subjected to SDS-PAGE and transferred onto

membranes. Membranes were blocked in 5% milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with primary monoclonal antibody against Rac1 (1:2000) (Clone 23A-8, Millipore, Lake Placid, N.Y.) followed by peroxidase-conjugated secondary antibody. Bands were visualized using SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Tubule formation assay

96-well plates were coated with 50 µl Matrigel™ (BD Biosciences, San Jose, CA). 48 hr after transfection, HUVECs were seeded at 1.0 x 10^4 cells per well in Opti-Mem (Invitrogen), supplemented with 10 ng/ml recombinant human $VEGF_{165}$ (R&D Systems, Minneapolis, MN) and 0.1% gentamycin, or EGM-2. Cells were incubated for 4-5 hr, after which wells were photographed at 40x magnification with a Nikon TE2000 microscope. Tubule formation was quantified by counting the number of branching points and measuring the total length of capillary tubes in at least three images using NIH ImageJ software.

Scratch wound assay

48 hr after transfection, HUVECs were seeded at 1.0 x 10^5 cells per well in 24-well plates in EGM-2 and allowed to grow to 100% confluency for 24 hr. Cells were washed with PBS and serum-starved for 1 hour with Opti-Mem supplemented with 0.1% gentamycin. A scratch was induced with a pipette tip, cells were washed 3 times with PBS and fresh Opti-Mem supplemented with 0.1% gentamycin and 10 ng/ml recombinant human $VEGF_{165}$ was added. Migration was checked after 24 hours. Scratch wound size was analyzed using NIH ImageJ software.

Invasion assay

48 hr after transfection, 0.5 x 10^5 HUVECs in 0.5 ml Opti-Mem supplemented with 0.1% gentamycin were added to BD Biocoat Matrigel Invasion Chambers, pore-size 8µm (BD Biosciences). Inserts were placed in 24 well plates containing 0.75 ml Opti-Mem supplemented with 0.1% gentamycin and 20 ng/ml recombinant human $VEGF_{165}$ as a chemoattractant and allowed to invade for 24 hours. After 24 hours, inserts were removed from well plates and medium was removed from the inserts which were then washed with PBS. Inserts were placed in 10% formalin for 10 minutes and left to dry. H&E staining was performed on cells on the bottom side of the inserts. Membranes were fixed on a glass microscope slide, scanned and cells present on the filter were counted.

Proliferation assays

Cell proliferation was measured using the sulforhodamine B (SRB) colorimetric assay (16). 24 hr after transfection, 4.0 x 10^3 cells per well were seeded in 96-well plates. At

indicated time points, cells were fixed in 4% trichloroacetic acid for at least 1 hr at 4°C, washed and stained with 0.4% SRB in 1% acetic acid for 30 min followed by air drying. Finally, bound dye was solubilized in 200 µl 10 mM Tris for 15 minutes. OD values were read at 490 nm.

Matrigel plug assay

All animal experiments were performed according to Dutch national regulations and approved by the local animal experiments ethical committee. Six to eight-week old male A/J mice (Harlan) were injected subcutaneously with 400 µl High Concentration Matrigel (BD Biosciences) supplemented with either $VEGF_{165}$ (0.3 µg/ml) or Neuro2a cells (2 x 10^6 cells/ml). On day 1 and 4 after injection, mice were treated locally with 10 µg siRNA, followed by electroporation (200V/cm, 2x2 pulses at perpendicular angles, 100 mS) under anesthesia. On day 7, mice were sacrificed and plugs were removed. Plugs were photographed, homogenized in RIPA buffer on ice followed by removal of debris by centrifugation. Hemoglobin content was determined using the QuantiChrom™ Hemoglobin Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions.

***In vivo* tumor model**

Six to eight-week old male A/J mice (Harlan, The Netherlands) were subcutaneously injected with 100 µl Neuro2a cells (1 x 10^7 cells/ml). Tumor sizes were measured using digital calipers and calculated using the formula: Size = length x $width^2$ x 0.52. Mice with established tumors (approximately $100mm^3$) were treated intratumorally on day 0, 2, 4 and 6 with 10 µg of negative control or Rac1 siRNA followed by electroporation. On day 7, mice were sacrificed and tumors were excised. For determination of Rac1, CD31 or VEGFR-2 levels, tumors were homogenized in RIPA buffer on ice, allowed to stand for 30 min followed by removal of debris by centrifugation. Protein levels were determined by Western Blot analysis as described, using a polyclonal antibody against CD31 (1:500) (Clone M-20, Santa Cruz Biotechnology, Santa Cruz, CA) or a monoclonal antibody against VEGFR-2 (1:1000) (Clone 55B11, Cell Signaling Technology, Denvers, MA). For immunohistochemical staining, frozen sections were fixed in acetone, rehydrated and blocked in 5% normal goat serum. For detection of CD31, sections were incubated with primary polyclonal antibody against CD31 (1:500) followed by biotinylated secondary antibody and HRP-conjugated streptavidin complex. Sections were stained with 3-amino-9-ethylcarbazole (AEC). Microvessel density (MVD) was quantified by counting the positively stained luminal structures in three representative images per animal. For Ki-67 detection, sections were incubated with primary polyclonal antibody against Ki-67 (1:300) (Ab66155, Abcam, Inc, Cambridge, MA) followed by poly-HRP goat anti-rabbit IgG (BrightVision, Immunologic, Duiven,

The Netherlands). Sections were stained with 3,3'-diaminobenzidine (DAB). The number of Ki-67-positive cells was counted in two representative images per animal. In all staining procedures, endogenous peroxidase activity was blocked using 0.3% H_2O_2/PBS and sections were counterstained using hematoxylin.

Statistical analysis

To assess statistical significances, Student's t-tests were performed. For multiple comparisons, ANOVA with Bonferroni post-tests was used.

RESULTS

Electroporation-mediated transfection of siRac1 silences Rac1 protein in HUVECs

HUVECs were transiently transfected using electroporation. To optimize the electrical parameters, cells were transferred into electroporation cuvettes and electroporated for 70ms at a voltage of 160V and 180V, using siRNA (450 and 700 pmol). After two days, silencing was determined using Western Blot analysis. Electroporation at a voltage of 180V using 700 pmol siRNA resulted in effective knockdown of Rac1 protein. This effect was specific for siRac1, as electroporation at the same settings and siRNA dose using siNS did not induce silencing (Figure 1). For further experiments, cells were treated using these optimized settings. Alternatively, where indicated, cells were transfected using Lipofectamine 2000, which also led to effective silencing of Rac1 protein (Figure S1).

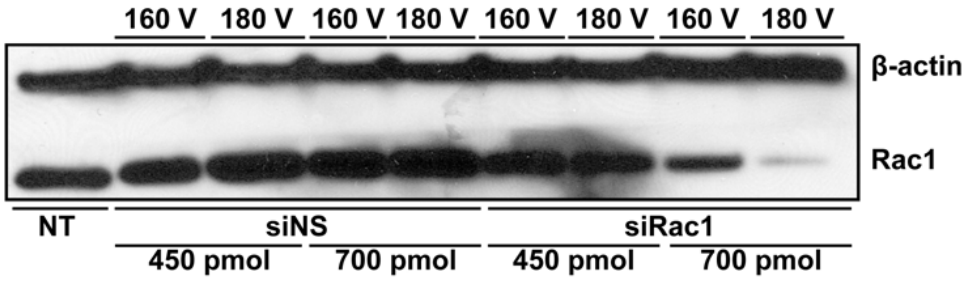

Figure 1. Knockdown of Rac1 in HUVECs. HUVECs were transiently transfected with siNS or siRac1 by electroporation. HUVECs were transfected with 450 or 700 pmol of siRNA per 1 * 105 cells using a single pulse of 160 V or 180 V. Silencing of the Rac1 protein was determined by Western Blot.

Rac1 silencing inhibits VEGF-induced tubule formation of HUVECs

To examine the effect of Rac1 silencing on HUVEC tubule formation, transfected cells were seeded on Matrigel in serum-free medium containing VEGF. HUVECs transfected with control siRNA comprehensively formed tubular structures. In contrast, cells

transfected with Rac1 siRNA formed considerably less tubular structures (Figure 2a). Quantification of tubule formation by counting the total tube length and number of branching points per sample revealed that transfection with siRac1 inhibited total tube length by ±70% and reduced the number of branching points by ±80% compared to transfection with siNS (Figures 2b,c). Similar results were observed with HUVECs transfected using Lipofectamine 2000 and seeded on Matrigel in complete medium (EGM-2) (Figure S2).

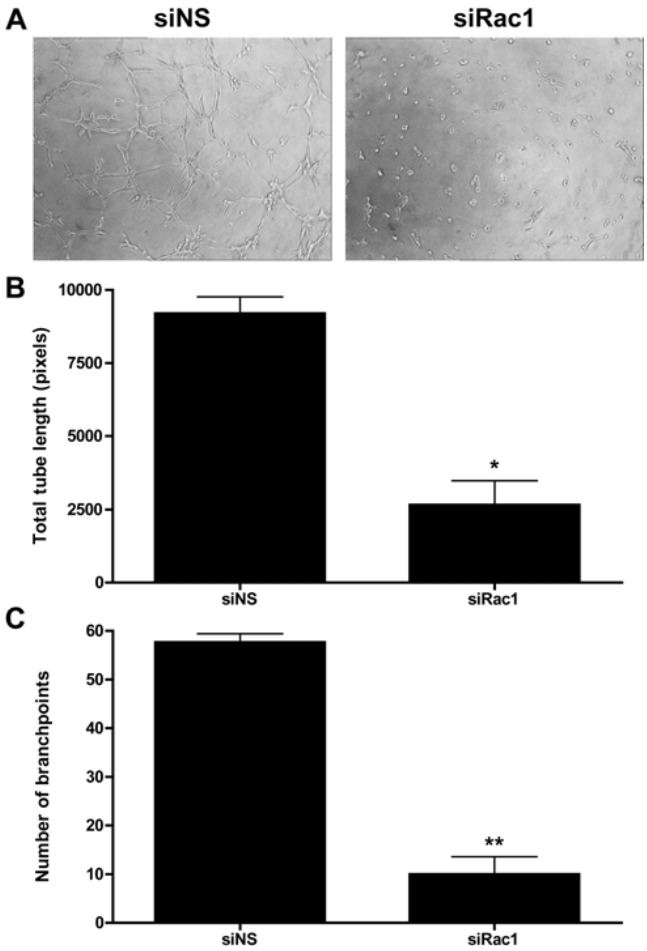

Figure 2. Rac1 silencing by siRNA reduces VEGF-induced tube formation of HUVECs on Matrigel. HUVECs transfected with siNS or siRac1 were seeded on Matrigel in Opti-Mem supplemented with 10 ng/ml recombinant human $VEGF_{165}$ and incubated for 4-5 hrs. Tube formation was visualized by microscopy at 40x magnification (a) and quantified by measuring the total length of capillary tubes (b) and counting the number of branching points (c) per picture. Data are presented as mean ± SEM of three individual experiments. *p<0.01, **p<0.001 vs. NS.

Rac1 silencing inhibits VEGF-induced migration and invasion of HUVECs

Rac1 is well known for its role in endothelial cell migration (17). To confirm that inhibition of Rac1 in HUVECs influences motility, a monolayer of transfected HUVECs was wounded and allowed to heal for 24 hours. While VEGF induced repair of the wounded monolayer of HUVECs transfected with siNS, monolayer repair was reduced for HUVECs transfected with siRac1 (Figure 3a). After 24 hours, VEGF-induced wound closure was 5-fold lower for Rac1-depleted HUVECs compared to control (Figure 3b).

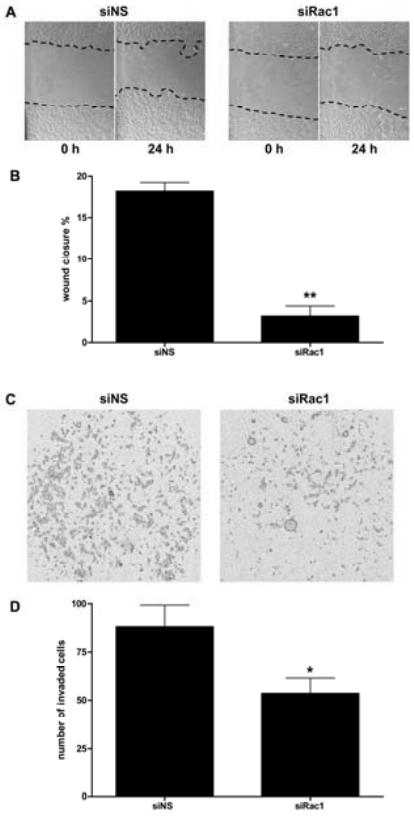

Figure 3. Rac1 silencing reduces VEGF-induced migration and invasion of HUVECs. HUVECs transfected with siNS or siRac1 were serum-starved, wounded and allowed to migrate in Opti-Mem supplemented with 10 ng/ml recombinant human VEGF165. After 24 hours, wound closure was visualized by microscopy at 40x magnification (a) and quantified using NIH ImageJ software (b). HUVECs transfected with siNS or siRac1 were added to BD Biocoat Matrigel Invasion Chambers in Opti-Mem, placed in 24 well plates containing 0.75 ml Opti-Mem supplemented with 20 ng/ml recombinant human VEGF165 and allowed to migrate for 24 hours. Filters were photographed (c) and invasion was quantified by counting the amount of invaded cells on the filter (d). Data are presented as mean ± SEM and are representative of 2 individual experiments. *$p<0.05$, **$p<0.001$ vs. NS.

To examine HUVEC invasiveness, transfected cells were allowed to invade through a Matrigel-coated filter in an invasion chamber, using VEGF as a chemoattractant in the lower chamber. The number of HUVECs that invaded through the Matrigel was almost two-fold lower for siRac1 transfected cells compared to siNS transfected cells (Figures 3c,d).

Rac1 silencing inhibits proliferation of HUVECs

The effect of Rac1 silencing on HUVEC proliferation was assessed using the SRB assay. Cells were transfected using Lipofectamine 2000. Cell counts of control transfected cells rose by approximately 80% following one and 300% following three days after seeding. In contrast, only a 30% and 180% increase during these periods of time was seen for siRac1-transfected cells (Figure 4).

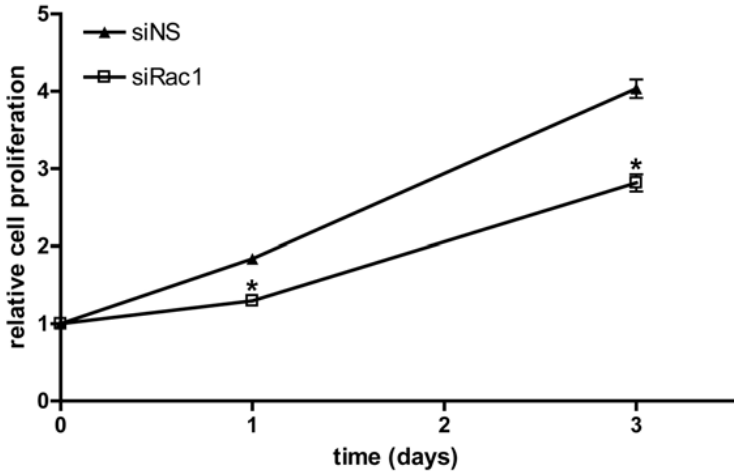

Figure 4. Rac1 silencing reduces proliferation of HUVECs. HUVECs transfected with siNS or siRac1 were seeded in 96-well plates. At indicated time points, cells were fixed and quantified using SRB assay. Data are presented as mean ± SEM and are representative of 3 individual experiments. *$p<0.05$ vs. siNS.

Rac1 silencing inhibits VEGF-induced angiogenesis *in vivo*

To investigate the inhibitory effect of Rac1 silencing on angiogenesis *in vivo*, a Matrigel plug assay was performed. Mice, subcutaneously inoculated with Matrigel supplemented with VEGF, were treated locally with siNS or siRac1. After 7 days, plugs were removed and examined for vessel formation. While plugs treated with siNS appeared red, siRac1-treated plugs were light-yellow, indicating reduced blood vessel formation and thus red blood cells, inside plugs. Matrigel plugs without VEGF appeared white/yellow (Figure 5a). Determination of hemoglobin concentrations in plugs showed a decrease in

hemoglobin content of siRac1-treated plugs versus siNS-treated plugs by almost 50% (Figure 5b). Comparable results were observed in an assay using Matrigel plugs containing Neuro2a cells, demonstrating the importance of Rac1 in tumor angiogenesis (Figure S3).

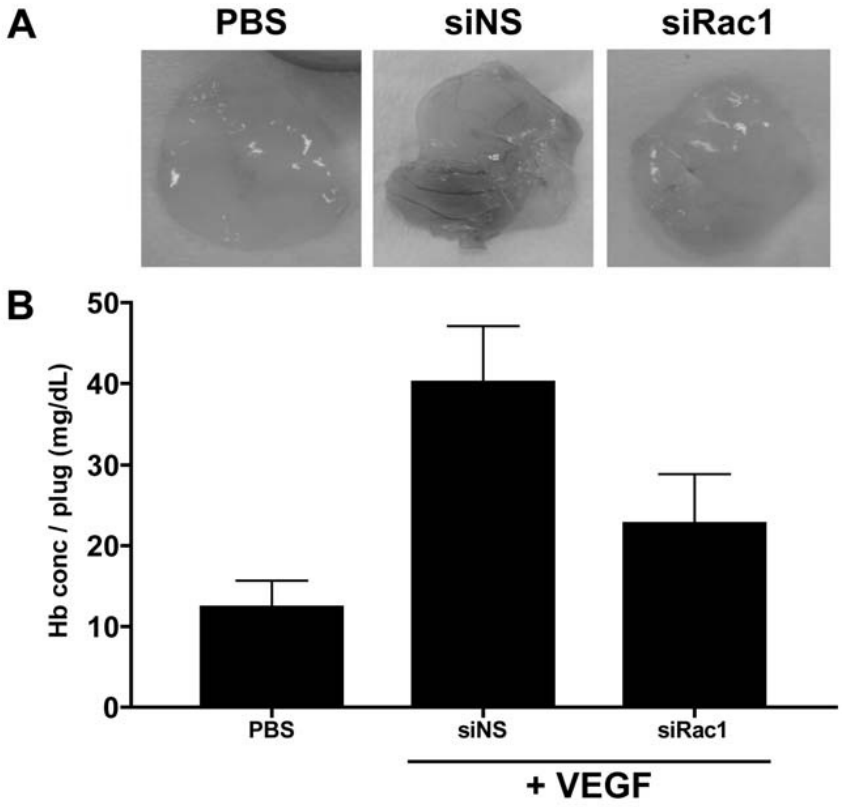

Figure 5. Rac1 silencing reduces VEGF-induced angiogenesis *in vivo*. Mice were injected subcutaneously with Matrigel supplemented with VEGF. On day 1 and 4 after injection, mice were treated locally with 10 µg siRNA. On day 7, mice were sacrificed and plugs were removed. New vessel formation was visualized by photography (a) and quantified by measuring the hemoglobin contents in Matrigel plugs (b). Data are presented as mean ± SEM. p=0.0865 for siRac1-treated vs. siNS-treated.

Rac1 silencing inhibits tumor growth and angiogenesis

The anti-tumoral and anti-angiogenic effects of Rac1 siRNA were investigated in subcutaneous Neuro2a neuroblastoma tumors. Established tumors (approximately 100 mm^3) were treated intratumorally every two days with 10 µg siRNA, followed by electroporation. As shown in Figure 6a, treatment with siRac1 almost completely

inhibited tumor growth, compared to treatment with control siRNA or no treatment. At day 7 following the first treatment, the average increase in size of tumors of siRac1-treated animals was 1.6-fold. In contrast, tumor sizes of untreated or siNS-treated animals increased 8-fold or 9-fold, respectively. Western blot analysis of Rac1-levels in tumors after sacrificing the animals on day 7 revealed efficient knockdown of Rac1 in tumors treated with siRac1 (Figure 6b).

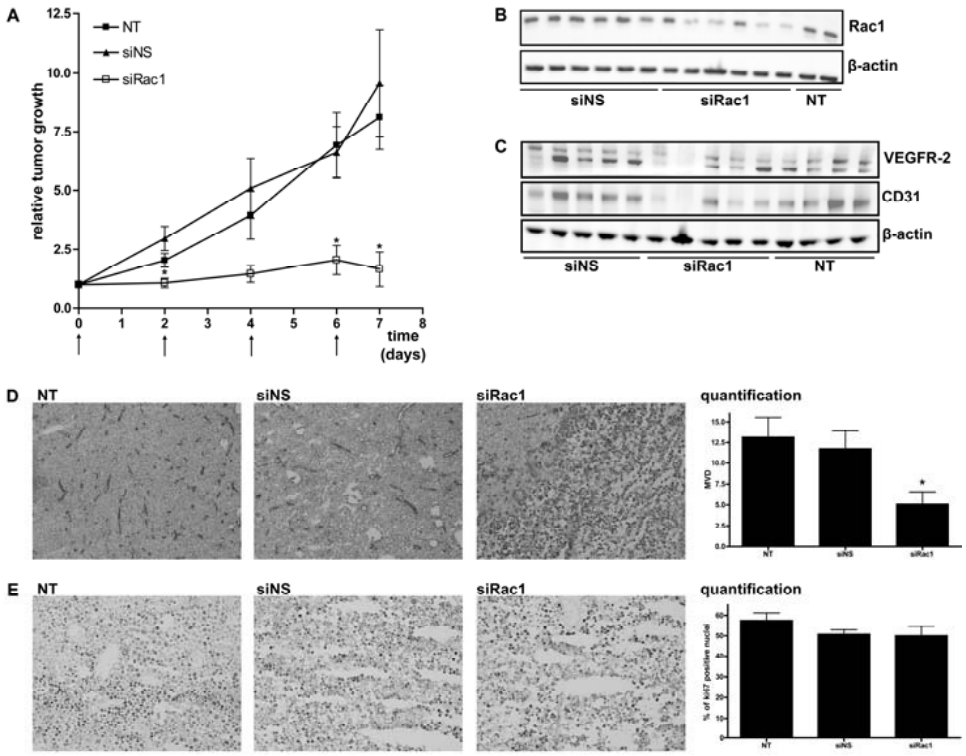

Figure 6. Local treatment of mice bearing Neuro2A tumors with siRac1 inhibits tumor growth and angiogenesis. Mice were inoculated subcutaneously with Neuro2A cells. After 7 days treatment was started. Mice were treated intratumorally on day 0, 2, 4 and 6 with 10 µg of siNS or siRac1 followed by electroporation. Tumor growth was measured for 7 days (a). On day 7, mice were sacrificed and tumors were excised. Knockdown of Rac1 in tumor lysates was confirmed by Western Blotting (b). Expression of the endothelial markers CD31 and VEGFR-2 in tumor lysates were determined by Western Blotting (c). Expression of CD31 (d) and proliferation marker Ki-67 (e) were determined by immunohistochemistry. Representative images are shown. Quantification of MVD was done by counting the positively stained luminal structures in three representative images per animal. The number of Ki-67-positive cells was counted in two representative images per animal. Data are presented as mean ± SEM. *p<0.05 vs. siNS.

To determine the effect of siRac1 on tumor angiogenesis, vascularization in the tumors was evaluated on day 7. First, tumors were analyzed for expression of two endothelial cell markers, CD31 and vascular endothelial growth factor receptor 2 (VEGFR-2), by Western blotting. Expression of both markers was reduced in siRac1-treated tumors, indicating a reduction in vessel density compared to tumors excised from untreated or siNS-treated mice (Figure 6c). CD31 expression was also determined by immunohistochemical analysis. Immunolabeling of tumor sections showed intense staining of untreated or siNS-treated tumors, while staining was less abundant in tumors treated with siRac1. Quantification of microvessel density by counting positively stained luminal structures revealed that siRac1 treatment significantly inhibited tumor angiogenesis, compared to siNS treatment. Furthermore, necrotic areas were observed in the centers of these tumors, which is also likely due to siRac1-mediated inhibition of angiogenesis (18) (Figure 6d).

Besides effects in endothelial cells, knockdown of Rac1 in tumor cells might also contribute to the overall anti-angiogenic effect by inhibiting the hypoxia-induced production of VEGF by tumor cells (19). Therefore, VEGF levels in the tumors were determined by Western blot analysis. As shown in Figure S4, treatment with siRac1 did not alter VEGF expression in tumors compared to controls. This result suggests that the observed inhibition of angiogenesis originates from a direct effect of siRac1 on endothelial cells and not from an indirect effect caused by decreased VEGF secretion by tumor cells.

Furthermore, to exclude the possibility that the observed inhibition of tumor growth was caused by siRac1-induced inhibition of proliferation of the grafted Neuro2a cells, we determined if Rac1 silencing had an effect on proliferation of Neuro2a cells *in vitro* and *in vivo*. siRac1 transfection had no effect on *in vitro* cell proliferation as compared to controls (Figures S5a,b). Moreover, staining of the proliferation marker Ki-67 in tumor sections showed no differences in expression between treatments, with moderate staining in the centre (Figure 6e) and intense staining in the periphery of the tumors (Figure S5c).

DISCUSSION

Rac1 is a key member of the Rho family of GTPases and is involved in several cellular processes including endothelial cell migration, cell cycle control and lumen formation (5, 7). As these processes are important for tumor angiogenesis, interference with Rac1 signaling may represent a novel approach to inhibit tumor neovascularization and subsequently tumor growth.

To inhibit signaling through Rac1, we implemented a clinically relevant siRNA approach to specifically knockdown expression of Rac1. Experimental delivery of siRNA into cells *in vitro* and *in vivo* was mediated using an established electroporation approach, which

has been extensively used for delivery of plasmid DNA and siRNA into target cells or tissue, including tumors (20). By means of electroporation, cellular plasma membranes are transiently destabilized by externally applied, localized and controlled electric fields, facilitating the entry of foreign molecules into cells and tissues. Several clinical trials are currently investigating electroporation as a drug delivery technology in humans, including several cases involving gene therapy via electroporation as applied in the oncology setting (21). Following optimization of the electrical parameters for efficient transfection in HUVECs, effects of Rac1 knockdown on angiogenic properties of cells were investigated. siRac1-transfected HUVECs demonstrated reduced ability to form capillary-like tubules as well as showing reduced migration, invasion and proliferation phenotypes compared to cells transfected with siNS. Reduced VEGF-induced tube formation and migration after Rac1 depletion in endothelial cells is in line with other studies (9, 22-24). Nevertheless, controversy exists regarding the role of Rac1 in endothelial cell invasion and proliferation. In agreement with our observations, silencing of Rac1 decreases HUVEC invasion through a fibronectin-coated Transwell filter (23). In contrast, Rac1-depleted mouse lung endothelial cells have been shown to invade Matrigel-coated Transwells to the same degree as controls (22). In the same study, no effect of Rac1 silencing on VEGF-induced proliferation was found. A possible explanation for this apparent discrepancy could be differences in cell type and growth conditions (e.g. serum concentration (25)). However, more studies are necessary to shed light on this issue.

To evaluate the effect of Rac1 knockdown on angiogenesis *in vivo*, a Matrigel plug assay was performed. Intratumoral injections of Rac1 siRNA resulted in decreased infiltration of endothelial cells in VEGF-containing plugs, indicating an important role of endothelial Rac1 in VEGF-induced angiogenesis. In our Neuro2a xenograft model, the inhibitory effect of siRac1 on tumor angiogenesis was also likely to be a result of its direct effect on endothelial cells. The importance of endothelial Rac1 for angiogenesis *in vivo* has been suggested by several other studies. For example, endothelial-specific Rac1 haploinsufficient mice display impaired eNOS activity and angiogenesis in a hind limb ischemia model and aortic capillary sprouting assay (26). In addition, transduction of endothelial cells with active L61Rac1 improves VEGF-mediated neovascularization and lumen formation *in vivo*, while transfection with dominant-negative N17Rac1 inhibited formation of vessels (27). In contrast, D'Amico et al. demonstrated that inducible deletion of Rac1 in wild-type endothelial cells does not affect tumor angiogenesis or VEGF-mediated angiogenesis in mice, unless β3-integrin is absent (22). The apparent difference in results between these studies may arise from differences in experimental model and setup.

Besides inhibiting angiogenesis, our data show that intratumoral injections of siRNA against Rac1 almost completely block the growth of established Neuro2a neuroblastoma

tumors. Similar results using RNAi were apparent for a different Rho GTPase, RhoA, in xenografted MDA-MB-231 breast cancer tumors (18). The authors suggest that the reduced tumor growth is the result of a combination of inhibition of proliferation of tumor cells and inhibition of angiogenesis. We did not find an effect of Rac1 silencing on proliferation of Neuro2a cells *in vitro* and *in vivo*, which makes it unlikely that reduced proliferation of grafted Neuro2a cells accounts for the observed reduction in tumor growth. However, we cannot exclude that, besides effects on angiogenesis pathways, inhibition of other Rac1-regulated processes in stromal cells may have contributed to the decrease in tumor growth.

Although several strategies to interfere with the process of angiogenesis have been therapeutically validated in both preclinical and clinical trials, recent studies have shown that anti-angiogenic therapy may paradoxically select for more invasive and metastatic tumor types (28-30). Combinatorial strategies that target proteins involved in both angiogenesis and invasion mechanisms might overcome this adaptive-invasive resistance. As several studies have indicated a crucial role for Rac1 in tumor cell migration and invasion, interfering with Rac1 signaling in both tumor- and endothelial cells could improve therapeutic outcome and provide a useful approach towards enduring and effective anti-cancer responses (31).

While current therapeutic strategies to inhibit Rac1 function such as lipid modification (32) and other small molecule inhibitors lack specificity, RNA interference technology may provide a means to specifically and efficiently inhibit Rac1 expression. However, the use of siRNA in the clinic is still hampered by ineffective delivery into target cells or tissues. Nevertheless, Davis et al. have recently demonstrated that RNA interference may be successfully employed in humans via systemically delivered siRNA, delivered using targeted nanoparticles, thus demonstrating that siRNA can be used as a gene-specific therapeutic (33).

As Rac1 is ubiquitously expressed throughout the body and controls a large number of cellular functions, interfering with Rac1 signaling could result in undesired side-effects (4, 34). Strategies to target tumor-associated cells without affecting normal cells may offer a larger therapeutic window. As a number of targeted delivery systems that specifically deliver siRNA to tumor sides are currently under preclinical and clinical development (33, 35, 36), this approach may soon appear applicable.

In summary, we have shown that Rac1 is an important regulator of VEGF-induced angiogenesis in endothelial cells. Our data further suggest that inhibition of Rac1 using RNA interference is an effective tool for inhibiting angiogenesis and tumor growth. As Rac1 is also involved in tumor cell migration and invasion, siRNA-mediated Rac1 silencing in both tumor and tumor-associated endothelial cells using targeted delivery systems represents a promising therapeutic strategy.

ACKNOWLEDGEMENTS

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Supporting information

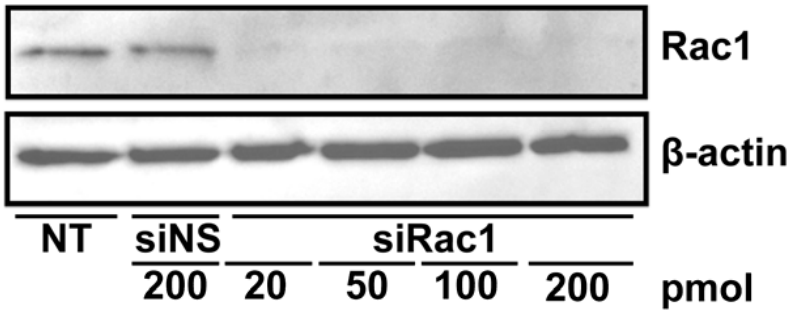

Figure S1. Knockdown of Rac1 in HUVECs. HUVECs were transiently transfected with siNS or increasing concentrations of siRac1 complexed with Lipofectamine 2000. Silencing of the Rac1 gene was determined by Western Blot analysis.

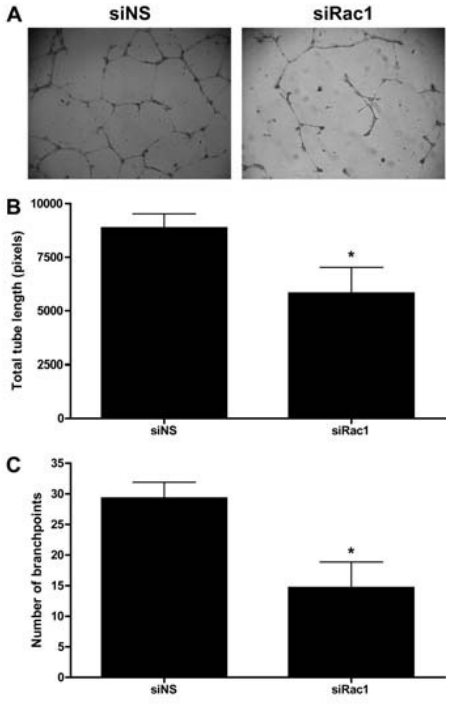

Figure S2. Rac1 silencing by siRNA reduces tube formation of HUVECs on Matrigel. HUVECs transfected with siNS or siRac1 using Lipofectamine 2000 were seeded on Matrigel in EGM-2 and incubated for 4-5 hrs. Tube formation was visualized by microscopy at 40x magnification (a) and quantified by measuring the total length of capillary tubes (b) and counting the number of branching points (c) per picture. Data are presented as mean ± SEM of three individual experiments. *$p<0.05$ vs. siNS.

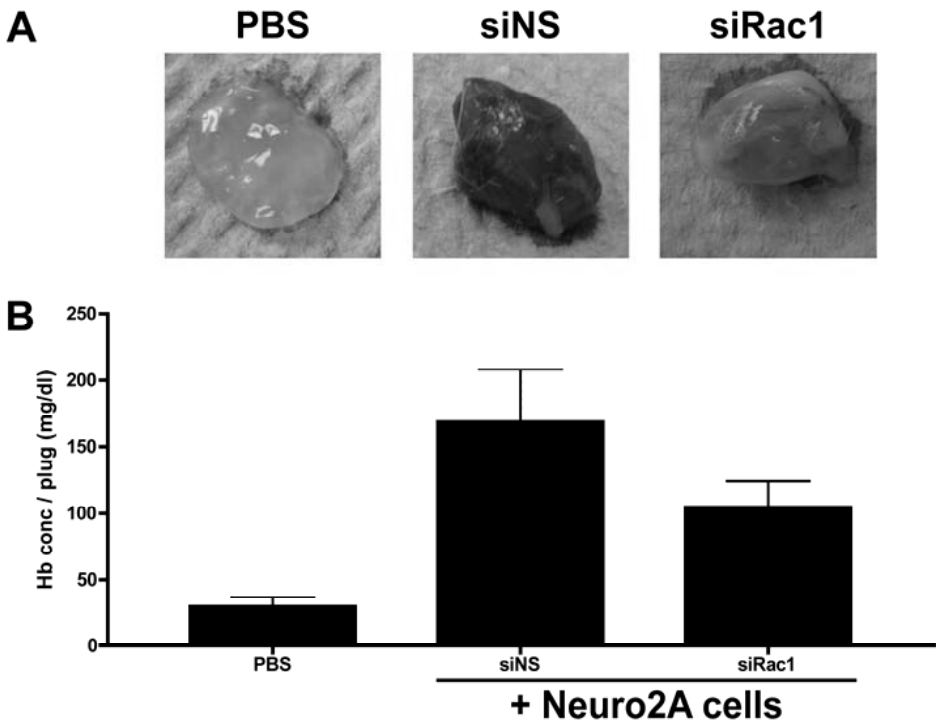

Figure S3. Rac1 silencing reduces Neuro2A-induced angiogenesis in vivo. Mice were injected subcutaneously with Matrigel mixed with Neuro2A cells. On day 1 and 4 after injection, mice were treated locally with 10 μg siRNA. On day 7, mice were sacrificed and plugs were removed. New vessel formation was visualized by photography (a) and quantified by measuring the hemoglobin contents in Matrigel plugs (b). Data are presented as mean ± SEM. p=0.1765 for siRac1-treated vs. siNS-treated.

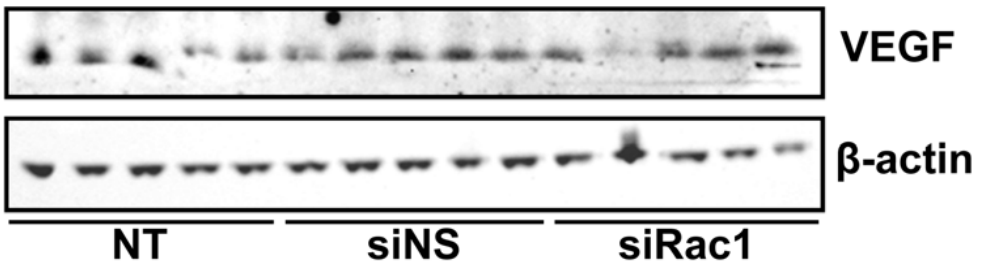

Figure S4. Rac1 silencing does not increase VEGF production in tumors cells *in vivo*. Tumors were homogenized in RIPA buffer on ice, allowed to stand for 30 min followed by removal of debris by centrifugation. VEGF levels were determined by Western Blot analysis as described, using a polyclonal antibody against VEGF (1:200) (sc-507, Santa Cruz Biotechnology, Santa Cruz, CA).

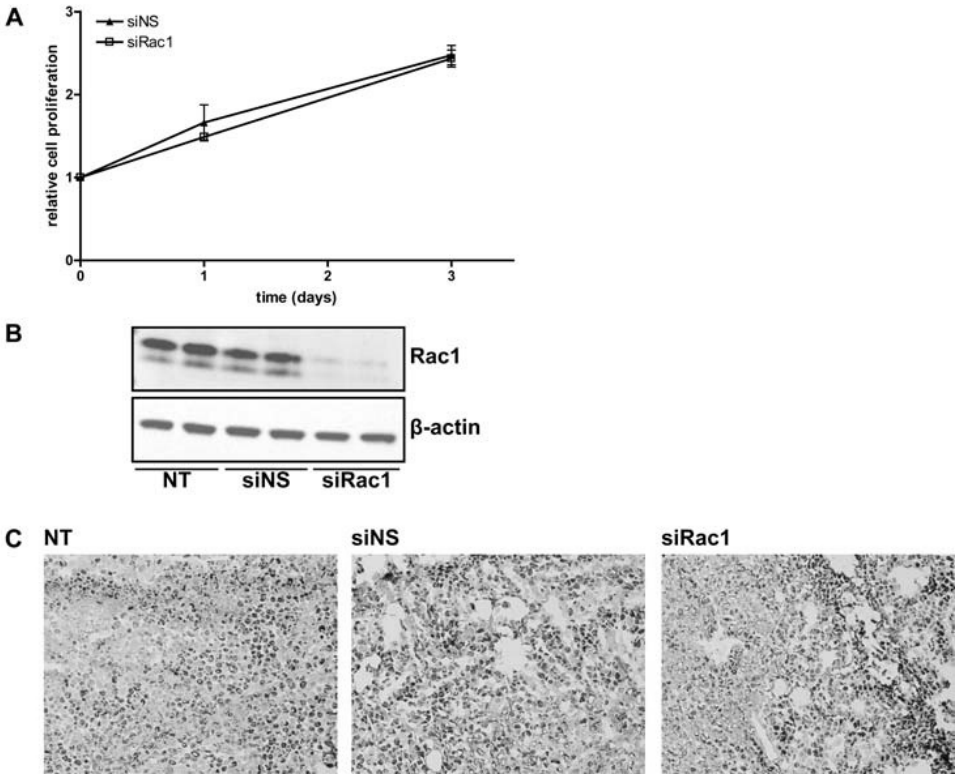

Figure S5. Rac1 silencing has no effect on proliferation of Neuro2a cells *in vitro* and *in vivo*. Neuro2a cells transfected with siNS or siRac1 were seeded in 96-well plates. At indicated time points, cells were fixed and quantified using SRB assay. Data are presented as mean ± SEM and are representative of 3 individual experiments (a). Knockdown of Rac1 in Neuro2a cells was confirmed by Western Blot analysis (b). Expression of proliferation marker Ki-67 in the periphery of tumors was determined by immunohistochemistry (c).

Chapter 3

Polymeric carrier systems for siRNA delivery

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ABSTRACT

RNA interference is a technique to induce sequence-specific gene silencing, but is hampered by inefficient delivery of its mediator, short interfering RNA, into target cells. This review describes recent advances in siRNA delivery using polymeric carrier systems. Structural variations that have been applied to these polymers for optimizing their intracellular trafficking are discussed, as well as strategies for stabilization and targeting to diseased tissues *in vivo*. Recent findings have highlighted safety issues that need to be taken into account in the design of nanoparticles for clinical application.

INTRODUCTION

RNA interference (RNAi) is a novel approach to induce sequence-specific gene silencing. Recently, RNAi has generated remarkable excitement in the biomedical research community, because of its applications in functional genomics, through study of loss-of-function phenotypes, as well as an emerging promise as a novel therapeutic. In order to realize the high expectations in therapeutic applications, a number of hurdles have to be overcome. First, cell membrane passage of a relatively large, strongly negatively charged molecule like siRNA presents a major obstacle. Furthermore, siRNA specifically needs to enter the cells that are actively translating the mRNA that is targeted in order to be effective. Finally, before it reaches these cells, unmodified siRNA has a half-life of mere minutes in biological environments, creating a need for protection. Various approaches for the delivery of siRNA have been proposed. Generally two classes of delivery systems can be distinguished, namely viral and non-viral carriers. Although viruses possess outstanding transfection efficacies, their inherent drawbacks are limited loading capacity, complicated large scale production and, most meaningful, severe safety risks due to immunogenicity after repeated administration. This gives them only limited potential for broad clinical applications (1). Non-viral delivery systems, which comprise cationic polymers and lipids, may circumvent some of the shortcomings of viruses. Although their efficiency is still inferior to their viral counterparts, substantial progress has been made in the last decade to develop and improve these delivery systems. In this paper we will focus on recent advances in siRNA delivery using polymeric carrier systems.

Polymers that have been explored to encapsulate and deliver siRNA can generally be divided in natural and synthetic polymers. Natural polymers include chitosan, atelocollagen and cationic polypeptides. Synthetic polymers comprise linear and branched poly (ethyleneimine) (PEI), poly-L-lysine (PLL), linear poly (amido amine)s (PAA) and poly (amido amine) dendrimers (PAMAM). Structural representations of these polymers are shown in Figure 1. Recent reviews provide a good overview of their performance (2-5), here we will zoom in on polymer design. We discuss the structural variations that have been applied to these polymers for optimizing their functioning and bringing them closer to (pre-)clinical utilization.

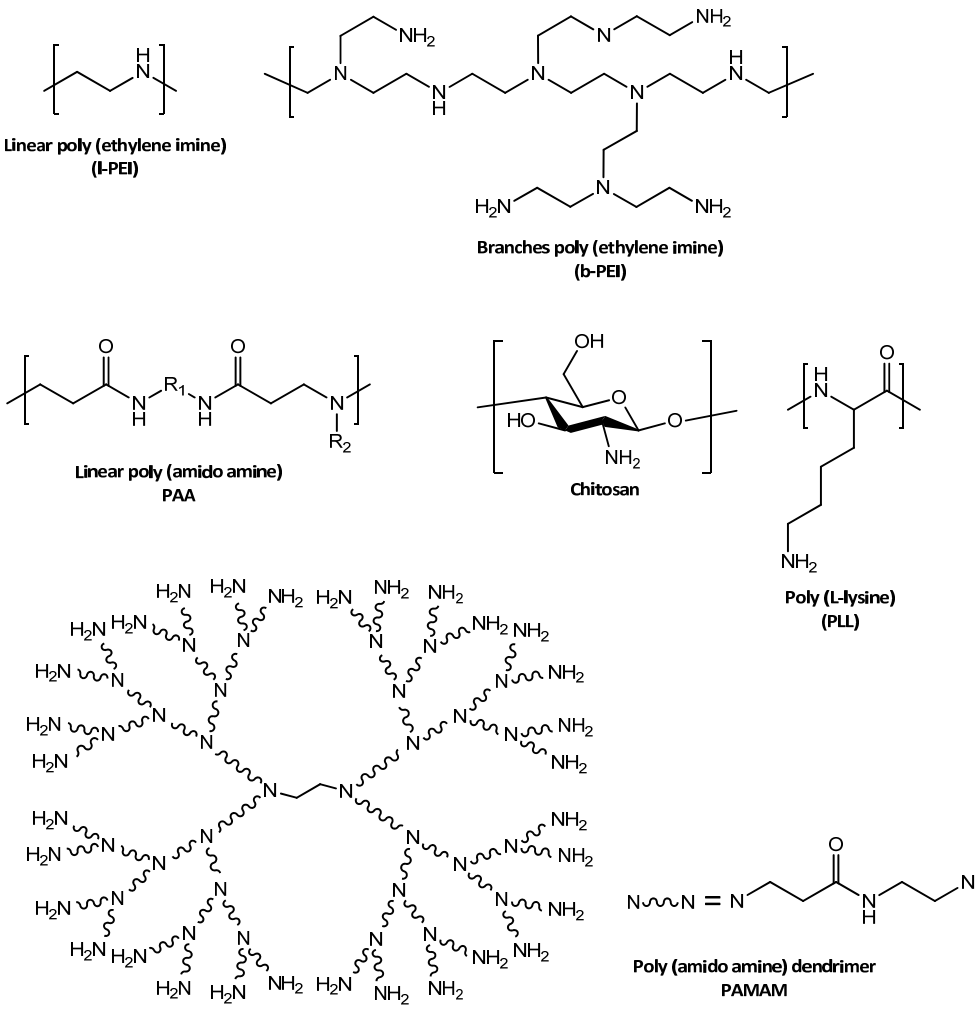

Figure 1. Structures of frequently used polymers for siRNA complexation.

CELLULAR DELIVERY

Formation of siRNA-polymer nanoparticles

The first polymeric systems that were investigated for siRNA delivery were derived from systems that have shown to be successful in plasmid DNA (pDNA) delivery. However, there are some fundamental differences between siRNA and pDNA, which makes an exchange of pDNA by siRNA not as straightforward as it might seem. The huge difference in number of nucleotides in pDNA (usually several thousand base pairs long)

and siRNA (only 21-27 base pairs), and therefore also the amount of negatively charged sites in these molecules, makes the interactions with polymers distinctly different. In pDNA-polymer complexes (polyplexes), many cooperative interactions can be formed between these two types of oppositely charged macromolecules with only limited loss of translational and conformational entropy in these macromolecules. Thereby, the condensed folding of the pDNA in these complexes provides many spatial possibilities to optimize DNA-polymer interactions. In contrast, siRNA molecules behave like rigid rods (6) and this absence of spatial flexibility poses high constraints on the formation of cooperative interactions with the polymer. Compared to pDNA, with siRNA less cooperative interactions with the polycationic polymer are possible and these interactions occur with higher loss of rotational (polymer) and translational (siRNA and polymer) entropy. These weaker intermolecular forces can lead to incomplete siRNA complexation or excessive size and poor stability of the formed complexes (7). Creative strategies have already been applied to use existing transfection reagents with siRNA. Complementary sticky overhangs of adenine and thymine were introduced by Behr et al. to reversibly concatemerize the siRNA molecules to make them gene like (ssiRNA) (8) and in the lipoplex field siRNA was co-complexed with long carrier DNA that serves as an entangler in the complex (9, 10).

The complex formation of siRNA and the polymer molecules is based on opposite charge attraction between the anionic nucleotide and cationic polymer. This process is favorable because of entropic gain upon the release of bound counterions (11, 12) and water molecules due to reduced hydration. The stability of the polyplexes can be improved by combining these coulombic interactions with for instance intercalation (13), hydrogen bonding (14) or hydrophobic interactions (15). Additional parameters like molecular weight and polymer architecture (linear, branched) which are known to effect the transfection process, do not seem to significantly influence the complexation processs (16, 17).

Cellular uptake of siRNA-polymer nanoparticles

After complexation, the formed polyplexes are taken up by the cells by endocytosis after interaction with the cellular membrane, which can be specific or non-specific. Specific interactions are usually receptor-mediated, which requires the polymer to be functionalized with a specific ligand for this receptor. Examples are functionalization with transferrin or folic acid for tumor targeting. Non specific interactions are generally based on electrostatic forces between the cationic polymer and negatively charged sulfated proteoglycans, like heparan sulfate, present on the cellular membrane (18).

After the initial binding, cells possess different endocytotic uptake pathways for non specific endocytosis, like clathrin and caveolae mediated endocytosis, macropinocytosis or phagocytosis (19, 20). However, cellular internalization routes are a turbulent research

field and it is at present absolutely not clear how siRNA complexes are exactly taken up. Studies investigating the uptake routes of polymeric siRNA particles are hard to find in literature. Only a few studies investigating the uptake of pDNA complexes have been reported, (21, 22) but it is difficult to draw general conclusions for siRNA complexes from those results.

Endosomal escape of siRNA polyplexes

When polyplexes are internalized, they end up in invaginations of the cell membrane. These small vesicles are called endosomes. Endosomes are transporter compartments, which either recycle the internalized molecules back to the cell surface or transform to late endosomes and finally fuse with lysosomes. Owing to the digestive function of the lysosomes, they have an acidic nature and contain several degrading enzymes. This milieu is not favorable for nucleotides. Therefore it is highly important that polyplexes (or their cargo) escape the endosomes, before externalization or degradation in the lysosomes. Different theories about possible escape mechanisms are currently pursued in literature. Although there is still some debate on the exact mechanism, there are strong indications that polymers which possess good buffering properties between pH 5 and 7.4 positively correlate with good endosomal escape properties.

An important theory about the action of the polyplex polymer in the endosomal escape process is the proton sponge hypothesis. This hypothesis was firstly postulated by Behr and coworkers, using PEI or a lipopolyamine (23, 24). According to this theory, the (unprotonated) basic sites of the cationic polymers act as buffer moieties by taking up the protons that are pumped in the endosomes on the way from endocytosis towards fusion with lysosomes. As a result of this pH buffering by the polymer, the endosomal ATPases have to pump in extra protons to reach the lysosomal pH. This proton influx simultaneously leads to an influx of chloride counterions. As a consequence of the increased osmolarity, an osmotic swelling occurs which is believed to cause the rupture of the endosomal membrane, releasing the polyplexes into the cytosol.

Although it offers a reasonable explanation for the endosomal escape of polymers that buffer in the endosomal pH range, the proton sponge theory remains under debate. Funhoff et al. measured a decrease instead of an expected increase in gene expression by improving the buffer capacity of pDMAEMA with the incorporation of an extra amine in the side chain (pDAMA) (25). Godbey et al. monitored the pH of the endosomes and lysosomes with fluorescent pH indicators in PEI-mediated transfection studies (26). Based on absence of pH change in lysosomes they concluded that there was no evidence for a proton sponge escaping mechanism. Oppositely, Sonawane demonstrated a high chloride accumulation after endocytosis of the buffering polymers PEI and PAMAM and a sharp decrease of chloride after one hour, suggesting a release of the polyplexes as

postulated in the proton sponge hypothesis (27). In addition, PLL, which is a poor buffering polymer, did not show high chloride accumulation.

Besides endosomal swelling others have argued that cationic polymers cause lysis of endosomal membranes. Fischer et al. showed the membranolytic ability of several frequently used cationic polymers by LDH release from L292 mouse fibroblasts(16). Both PEI and PLL showed a LDH release of almost 50% for low polymer concentrations (0.01 mg/ml) over 1 hour. In contrast, PAMAM and amine modified dextran did not show significant LDH release, even at 1 mg/ml. Similar behavior for these polymers was observed by Hong et al. in KB and Rat 2 cells (28).
Xiong modified a biodegradable amphiphilic poly(ethylene glycol)-*block*-polyester with three different polyamines (spermine (SP), tetraethlyenepentamine (TP), *N,N*-dimethyldipropylenetriamine (DP)) to introduce cationic charge and endosomal escape properties (Figure 2) (29).

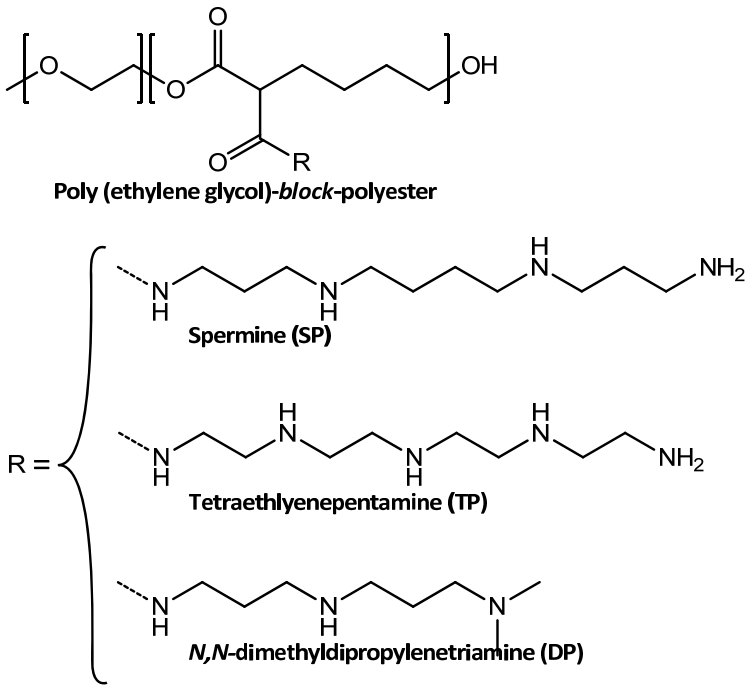

Figure 2. General structure of polyamine grafted poly (ethylene glycol)-*block*-polyester as they were prepared by Xiong (29). R represents the polyamines spermine (SP), tetraethlyenepentamine (TP) or *N,N*-dimethyldipropylenetriamine (DP).

All materials formed small polyplexes, but only the polymers possessing side chains with terminal primary amines yielded silencing. This was attributed to the ability to penetrate membranes at low pH. Knockdown values of 50 – 60% were achieved by silencing P-glycoprotein on MDA435/LCC6 tumor cells and were comparable with b-PEI. The endosomal escape for SP and TP modified polymers occurred in a similar time span. However the authors did not clarify if there was a difference in buffer capacity or lytic activity at low pH, so the successful endosomal escape cannot be assigned exclusively to one of those parameters.

Fusogenic peptides are sometimes used to enhance endosomal escape. However, these studies have mainly focused on siRNA-peptide conjugates, although a few examples have been described of such systems in combination with polymers.

HGP, a lytic peptide from the endodomain of HIV envelope gp41, was coupled to branched PEI with a ma;ecular weight of 25 kDa by Kwon et al. (30) Increased silencing of GAPDH in HeLa cells was reported for HPG-modified PEI (82%) in contrast to non-functionalized PEI (53%). Complex internalization, analyzed with fluorescently labeled complexes, was identical for modified and unmodified PEI, but confocal images showed a lower extent of colocalization of the HGP-modified complexes with endosomes after 3 hours. These results illustrate that HGP-PEI specifically penetrates endosomal membranes.

The membranolytic melittin, a major bioactive component of bee venom, was used ingeniously by Meyer et al. (31). Toxic extracellular membrane interactions were eliminated by protecting the primary amines on melittin with dimethylmaleic anhydride (DMMAn). This pH-labile protecting group is released upon acidification in the endosomes, resulting in a reactivation of the membranolytic capability of the peptide. The protected melittin was coupled reversibly to PLL-PEG and branched PEI-PEG copolymers via a disulfide linker (Figure 3).

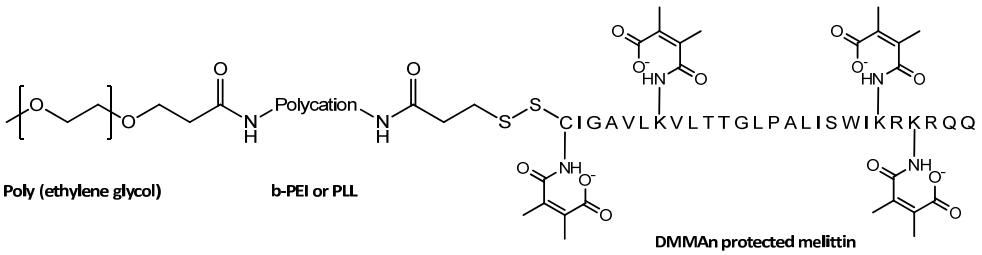

Figure 3. Schematic structure of melittin functionalized polycation (PEG-b-PEI or PEG-PLL). The dimethylmaleic anhydride protecting groups are released upon acidification, restoring the lytic activity of melittin after endocytosis.

Melittin modified polyplexes greatly enhanced the luciferase knockdown in luciferase expressing Neuro 2A-eGFPLuc cells. PEI-PEG-DMMAn-Mel achieved 60% knockdown versus 20% for PEI –PEG and 50% for PEI only. PLL-PEG-DMMAn-Mel accomplished even 90% knockdown, where PLL –PEG and PLL only did not show any significant knockdown. In a later study of the same group the PLL-PEG-DMMAn-Mel system appeared to dissociate in presence of low concentrations of heparin (32). Reversible conjugation of the siRNA to the PLL via a disulfide linker prevented this dissociation and did not affect the silencing properties.

siRNA release from polyplexes

Since polyplex formation is a spontaneous process that is entropically favorable by the release of bound water molecules and counterions it is unlikely that the complexes will dissociate spontaneously after endosomal escape. However, the cytosolic environment plays a major role in vector unpacking. PEI and dendritic PLL polyplexes were exposed to cytosolic liquid by Okuda et al. (33). The polyplexes dissociated at low polymer concentrations and this was attributed to interactions of the polymers with cytosolic proteins. Huth and coworkers performed comparable experiments, and concluded that interactions with cytosolic RNA were mainly responsible for the dissociation (34). Irrespective of the compounds causing polyplex dissociation, the unpacking is relatively slow and to a certain degree reversible. Release of siRNA by polymer degradation can be much faster and is irreversible. It has been shown that incorporation of hydrolysable or reducible linkers in the polymer chain can boost transfection efficiencies significantly.

The disulfide moiety is one of the most popular reducible linkers and this group can be reductively cleaved by reaction with thiol reagents like the biologically active reducing agent glutathione. Since the glutathione concentration in the intracellular space is three orders of magnitude higher than in the extracellular environment (35), disulfide-containing polymers are relatively stable in the transport phase to the cell, but will be rapidly degraded in the effector phase within the cytosol. An additional advantage of the separation into small polymer fragments is the decreased cytotoxicity of the polymer (36). We have extensively explored in our group the effects of disulfide linkages in poly(amido amine)-mediated gene delivery (13, 37-39), and recent results show that these versatile polymers can also be engineered for siRNA delivery (L.J. van der Aa et al., unpublished data). A reducible branched PEI (SS-PEI) was synthesized by Breunig by introducing disulfides via crosslinking linear low molecular weight PEI (2.6 kDa) with 3% Lomant's reagent (40) (Figure 4a). Silencing ability of this polymer was measured by knocking down EGFP in CHO-K1 cells. Although the silencing was slightly lower than with branched PEI (55% versus 75%), confocal laser scanning images indicated that SS-PEI indeed promotes the intracellular release of siRNA. Furthermore, significantly lower toxicity was observed for SS-PEI compared to non reducible b-PEI.

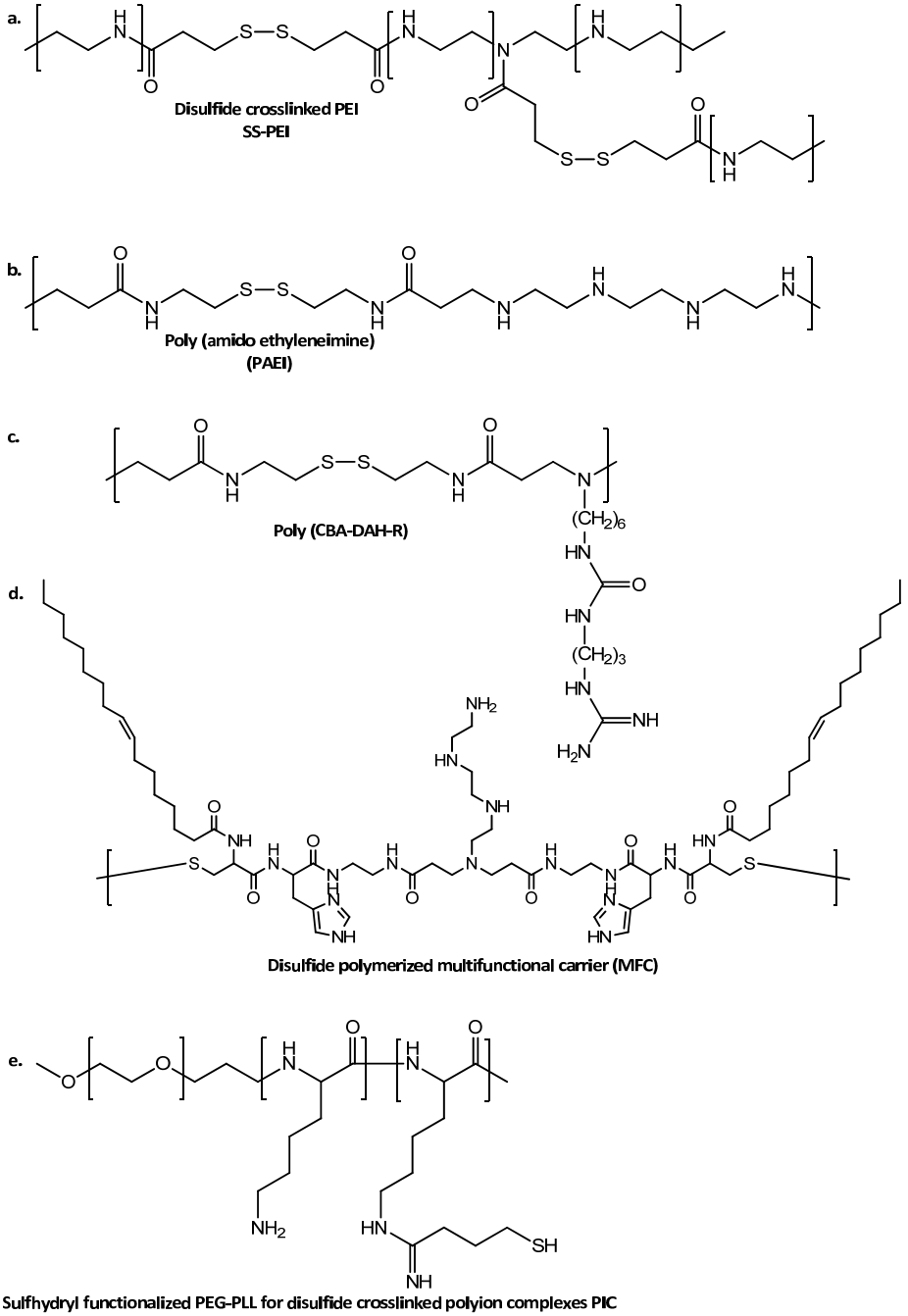

Figure 4. Disulfide containing polymers to promote cytosolic degradation upon glutathione mediated disulfide reduction.

A poly (amido ethyleneimine) PAEI was used by Jeong to have PEI-like structures in a reducible amide backbone (Figure 4b) (41). VEGF in PC-3 cells was targeted in the *in vitro* knockdown studies. At w/w ratio 6 and 12, the concentration of secreted VEGF in the supernatant was reduced from 700 pg/ml to approximately 150 pg/ml, whereas l-PEI only effectuated a reduction to 350 pg/ml. The intracellular release was imaged by confocal microscopy and was shown to be significantly better for PAEI than for l-PEI. Also the cell viability was conspicuously higher for PAEI at all measured concentrations. Similar results were obtained by the same group with an arginine modified disulfide based poly (amido amine) (poly (CBA-DAH-R), Figure 4c) (42). Polyplex dissociation could be nicely visualized by confocal microscopy and knockdown of VEGF in PC-3, KB, HeLa, A2780 and A549 cells showed similar efficiencies in all cells. Additionally, the toxicity profile of this polymer is highly advantageous for PAEI, since it does not show any toxicity at polymer/siRNA w/w ratio of up to 60/1.
Wang et al. reported a polymer that was polymerized by disulfide formation, which they named a multifunctional carrier (MFC) (Figure 4d) (43). Silencing was compared to DOTAP instead of PEI, due to the lipidic character of the MFC. Luciferase was knocked down to 40% of its original level against 60% for DOTAP. siRNA release from the carrier upon exposing the polyplexes to a reductive environment was unfortunately not reported, but in earlier work similar polyplexes with DNA proved to be reducible in presence of DTT (44).
The group of Katoaka established stable disulfide crosslinked polyion complexes (PIC) (45). PEG-PLL was treated with Traut's reagent to introduce sulfhydryl moieties on the primary amines of PLL with different degrees of thiolation (Figure 4e). The siRNA complexes were crosslinked using DMSO after complexation. The optimal thiolation degree to form stable particles turned out to be 14%. Luciferase knockdown in presence of serum was 100 times more efficient with these polyplexes than with their uncrosslinked analogues. The difference was attributed to the increased extracellular stability of the polyplexes created by the disulfide crosslinks.

Another possibility to enhance the release of siRNA by intracellular degradation of the polymer is by introducing acid-sensitive linkers in the polymer. The acid catalyzed hydrolysis of the linkers is induced by the acidification of the endosomal compartment during the endosomal maturation, which could lead to (partial) premature release of siRNA. This should not be a problem as long as the siRNA's do not end up in the lysosomes. Typical biocompatible pH-labile linker groups are acetals, ketals, orthoesters and hydrazones. They have been occasionally applied for gene delivery, though recently they have been investigated more frequently. Thus far, pH-labile linkers have been merely used for detaching PEG coatings, but recently the groups of Wagner and Kwon also used ketalized PEI to prepare polyplexes that destabilize in acidic environment.

Kwon et al. functionalized primary amines of LMW branched PEI (800 Da) and HMW branched PEI (25 kDa) at several degrees of ketal modification. The ketalization was performed by activation with para-nitrophenyl chloroformate, followed by a substitution with diamine dimethylketal (46, 47) (Figure 5a). The rationale for this modification was twofold: the reduction in the amount of primary amines would mitigate the cytotoxicity of PEI and, moreover, would weaken the interaction of the polymer with siRNA, thereby facilitation release of siRNA from the polyplex. Half-lives at pH 5.0 of hydrolytic degradation of the ketal linkers ranged between 2 and 5 hours, depending on the degree of ketalization. Gel electrophoresis experiments showed that LMW and HMW ketalized PEI were able to complex siRNA and release it upon hydrolysis up to N/P 40. TEM micrographs of swollen complexes after hydrolysis confirmed these observations. LMW ketalized PEI did not produce any silencing, since all of these complexes were localized mainly in the nucleus. HMW ketalized PEI on the other hand effectuated almost 80% knockdown of eGFP in NIH 3T3 cells. A higher N/P ratio was needed for ketalized HMW PEI than for unmodified PEI to obtain similar results. On the other hand, the ketalized polymers did not show any toxicity, whereas PEI is extremely toxic above N/P 20. The degree of ketalization for optimal silencing appeared to be 23% of the primary amines.

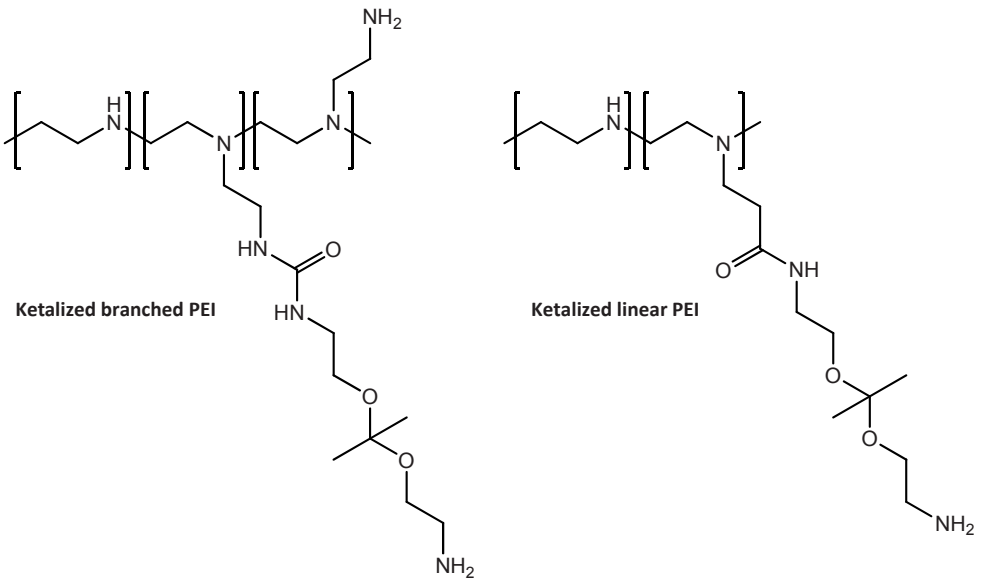

Figure 5. Acid labile dimethyl ketal grafted primary amines on branched (a) and linear PEI (b) to destabilize the siRNA complexes after endocytosis.

The same group also investigated the effect of attaching primary amino groups via a ketal spacer to linear PEI (Figure 5b) (48). LMW (2.5 kDa) and HMW PEI (25 kDa) were

reacted with acrylamide-functionalized ketal linkers with a ketalization degree of 22 and 24 mol%, respectively. Hydrolysis of the ketal functions at pH 5.0 proceeded with half lives around 2 hours. HMW ketalized PEI formed slightly larger polyplexes than non-modified PEI. Again, polyplex dissociation after hydrolysis was proven by gel electrophoresis and TEM. Confocal laser scanning micrographs revealed cytosolic dissociation of the ketalized polyplexes, whereas non-functionalized polyplexes were present in the nucleus. eGFP silencing efficiencies of 75% were obtained in NIH 3T3 cells with ketalized LMW PEI in absence as well as in presence of serum with cell viabilities of 80%. Comparison with polyplexes using PEI with a non-degradable linker to the primary amine functions confirmed that the improved silencing properties were indeed caused by the acid labile ketal linker.

IN VIVO DELIVERY

For therapeutic application of siRNA, intracellular delivery is not the only challenge. For effective therapy without side effects, specific delivery of siRNA to the target site is necessary. Local administration of siRNA avoids many of the difficulties associated with distribution of siRNA to non-target sites. However, many tissues can only be reached via the bloodstream, which requires systemic administration of the formulation.

Local administration

Several tissues are accessible for local administration of siRNA, including the eye, skin, muscles, lungs, brain and superficial tumors (49). Although successes have been described (50, 51), administration of naked siRNA usually does not result in silencing because of the inability of the siRNA to reach the target cells and cross the cellular or organelle membrane. As we have seen, complexation by polymers can facilitate uptake into cells, but, as we will discuss in this section, it can also protect against the harsh environment *in vivo* during the transport phase.

Among the cationic polymers, polyethyleneimine (PEI) has been most widely used for siRNA delivery *in vivo*. An interesting application of PEI has been recently described by Merkel et al. (52). In their study they showed, using actin-EGFP expressing mice, that intratracheal application of EGFP siRNA formulated with PEI 25kDa polyplexes leads to efficient EGFP silencing in the lung. The PEI polymers were first grafted with polyethylene glycol (PEG), which increased protection and residence times in the lung of the PEG-PEI/siRNA complexes compared to PEI/siRNA complexes. In another example, branched PEI (25kDa) was conjugated to hyaluronic acid (HA), an anionic biopolymer involved in wound healing, cell motility and angiogenesis (53). The authors hypothesized that coating of the PEI/siRNA polyplexes with HA would enhance serum stability and facilitate cellular uptake via HA receptor-mediated endocytosis. *In vitro*, siRNA/PEI-HA

complexes exhibited higher gene silencing efficiency than siRNA-PEI complexes, which was maintained in the presence of up to 50% serum. Furthermore, intratumoral injections of PEI-HA complexes containing anti-VEGF siRNA resulted in downregulation of VEGF in B16F1 tumors and effective inhibition of tumor growth. These systems could potentially be used for the treatment of diseases in tissues with HA receptors, such as liver cancer and kidney cancer (54).

The polysaccharide chitosan is a good candidate for drug delivery because of its biodegradability, biocompatibility and low immunogenicity (55). Chitosan-based systems have therefore also been investigated as siRNA delivery systems for different kinds of applications. Howard et al. introduced a chitosan-based siRNA nanoparticle delivery system for RNA interference *in vitro* and *in vivo*. Nasal administration of chitosan/siRNA particles resulted in approximately 40% silencing in bronchiole epithelial cells of transgenic EGFP mice, highlighting the potential application of these systems for treatment of mucosal diseases (56). In a study by De Martimprey et al., chitosan was used for coating of nanoparticles (57). They used a core-shell type of nanoparticles, where the core consisted of biodegradable poly(isobutylcyanoacrylate) polymer and the shell of positively charged chitosan. Intratumoral injection of nanoparticles containing siRNA against the fusion oncogene ret/PTC1 gave 82% silencing of the gene, resulting in significant inhibition of tumor growth. They also showed increased stability of nanoparticle-associated siRNA in tumors compared to free siRNA. Interestingly, because of their dual composition, these particles could additionally be loaded with hydrophobic compounds in the core, allowing for siRNA treatment in association with other anticancer molecules. Recently, chitosan nanoparticles were also used in cream for transdermal delivery of siRNA for the treatment of asthma (58).

Atellocollagen is a highly purified pepsin-treated type I collagen from calf dermis. It is low in immunogenicity and promotes cellular uptake and nuclease resistance of oligonucleotides (59). It has been most widely used for intratumoral delivery of siRNA in preclinical settings, including murine models of pancreatic cancer(60), HPV16+ cervical cancer (61) and non-seminomatous germ cell cancer (62).

Long-term, local, sustained release of siRNA has been investigated using poly(DL-lactic/glycolic acid) (PLGA) microspheres. In a study by Murata et al., anti-VEGF siRNA, together with arginine or branched PEI as transfection agent, was encapsulated in PLGA microspheres and its release in phosphate buffer (pH 7.4) was shown to be sustained for over one month. Intratumoral injection of these systems obviously suppressed tumor growth for over three weeks (63).

Systemic administration

Pharmacokinetics and biodistribution

Systemic administration of polyplexes is an attractive approach for delivery of siRNA to disseminated sites. While naked siRNA is rapidly degraded by serum nucleases *in vivo*, appropriate formulation of siRNA can prevent this and may increase its circulation time. Positively charged polyplexes, however, rapidly aggregate in the presence of salt or serum, which can lead to physical entrapment of the aggregated polyplexes within pulmonary capillary beds (64). Furthermore, opsonization of polyplexes leads to rapid clearance by the reticuloendothelial systems (RES), resulting in uptake in RES-rich tissues such as the liver and spleen (65).

The biocompatibility of polyplexes can be enhanced by modification of their surface with non-ionic polymers like poly (ethyleneglycol) (PEG). PEGylation of cationic polymers has been reported to reduce interaction with blood components and extend circulation time (66). In a recent study, Merkel et al. traced pharmacokinetics and biodistribution of intravenously administered siRNA polyplexes formed with PEI 25 kDa or PEGylated PEIs. They demonstrated that PEGylated polyplexes showed significantly less uptake in liver and spleen compared to PEI polyplexes (67). In another study from the same group, the *in vivo* pharmacokinetics and tissue distribution of a broad panel of PEI(-PEG)-based siRNA polyplexes after intravenous injection were investigated. They showed that the fate of the complexes mainly depended on the degree of uptake in liver, spleen, lung and kidney, and that the *in vivo* behavior of the polyplexes was determined by parameters such as siRNA complexation efficiency, complex stability in the presence of proteins, complex binding to plasma proteins and erythrocyte aggregation (68).

The tissue distribution of siRNA polyplexes can also be changed using targeting agents. In their study on PEG-coated poly(propyleneimine) dendrimer siRNA complexes, Taratula et al. showed that tumor targeting by a Luteinizing Hormone-Releasing Hormone (LHRH) peptide conjugated to the distal end of the PEG polymer significantly altered the fate of the dendrimer and siRNA. After systemic injection, non-targeted dendrimer and delivered siRNA were found mainly in the liver and the kidney, while only trace amounts accumulated in the tumor. In contrast, targeted dendrimer and delivered siRNA were predominately found in the tumor (69). In a study from a different group, however, it was claimed that both non-targeted and targeted siRNA nanoparticles exhibit similar biodistribution and tumor localization (70).

Disease modulation

During the last years, an increasing number of studies has been published addressing the therapeutic outcome of siRNA-administration *in vivo*. Again, linear or branched PEI based polyplexes have been most thoroughly investigated. PEI-complexed siRNA has been successfully used to deliver intact siRNAs into subcutaneous tumor xenografts after

intraperitoneal (i.p.) administration (71, 72). Using ^{32}P-labeled siRNA, Urban-Klein et al. showed that, compared to other organs, particularly strong siRNA accumulation was observed in the tumor at 30 minutes and 4 hours after administration. The authors contributed the preferential uptake in tumors to their high vascularization and the EPR (enhanced permeation and retention) effect. This passive accumulation in tumors after systemic administration has been described for many macromolecular agents and colloidal systems and is caused by defective vasculature combined with impaired lymphatic drainage observed universally for solid tumors (73). As the EPR-effect depends on the statistical probability of extravasation, accumulation improves with circulation time increase. Northern blotting revealed a ~50% reduction of the target gene (HER-2) in the tumors, which resulted in a significant reduction of tumor growth. I.p. injection of naked HER-2 siRNA failed to show any inhibitory effect (72). Although these results are promising, the applications of unmodified PEI polyplexes are limited, since they are prone to aggregation in the presence of serum. More recently, a new micelle-based delivery system for siRNA has been described (74). This system is based on the formation of polyelectrolyte micelles between PEGylated siRNA and branched PEI (25 kDa). The self-assembled, core-shell structures containing VEGF siRNA were used for anti-angiogenic therapy. Biodistribution experiments showed that 24 hours after intravenous (i.v.) injection, siRNA levels in PC-3 tumors were significantly higher for siRNA-PEG/PEI micelles than for siRNA/PEI formulations. For anti-angiogenic therapy, micelles were injected intravenously on days 0, 4, 10, 18 and 28 after tumors had reached 50 mm^3, which gave efficient VEGF silencing, leading to decreased microvessel density in tumors and significantly lower tumor volumes.

Delivery of polyplexes to a specific cell-type can be achieved using targeting agents. Actively targeted polyplexes were constructed by PEGylation of branched PEI polyplexes with an Arg-Gly-Asp(RGD) peptide ligand attached at the distal end of the PEG. RGD peptides are recognized by integrins, expressed by tumor neovasculature. Polyplexes were prepared using siRNA against vascular endothelial growth factor receptor-2 (VEGFR-2) and their uptake was found to be dependent on the presence of the ligand. After intravenous administration, selective uptake in tumors, siRNA sequence-specific inhibition of VEGFR-2 as well as inhibition of angiogenesis and tumor growth rate were shown (75). Tietze et al. used the serum protein transferrin (Tf) as both targeting and surface shielding agent for oligoethyleneimine (OEI) polyplexes. They showed that incorporation of Tf in the polyplexes prevented their aggregation and reduced their surface charge. Furthermore, systemic delivery of Tf-OEI polyplexes formulated with siRNA against RAN (three intravenous applications at 3 days interval) resulted in >80% silencing, apoptosis of neuro2A tumor cells and reduced tumor growth (76).

Atelocollagen has also been investigated for systemic delivery of siRNA. Inhibition of metastatic tumor growth in bone tissue was found by Takeshita et al. as a result of systemic treatment with siRNA against enhancer of zeste homolog 2 (EZH2) formulated with 0.05% atelocollagen (77). Intravenous injection of siRNA/atelocollagen complexes showed greater accumulation in tumor tissues compared with normal tissues, possibly due to the EPR mechanism. High amounts of siRNA were also detected in other tissues, including lung, spleen, kidney and liver. Indeed, intravenous injection of siRNA complexed with atelocollagen has also shown to be effective in the treatment of liver metastases of lung cancer in a mouse model (78). The EPR effect can also be exploited for targeting inflamed tissues. After systemic administration, atelocollagen-complexed siRNA was specifically delivered to inflammatory sites in a contact hypersensitivity (CHS) model in the mouse ear, without uptake in the non-inflamed contralateral ear. Atelocollagen facilitated the uptake of siRNA against monocyte chemoattractant protein-1 (MCP-1) into macrophages/monocytes and fibroblasts, which resulted in downregulation of MCP-1, inhibition of infiltration of inflammatory cells into the ear and suppression of ear swelling. The authors also compared their method to treatment with a liposome reagent and concluded that the atelocollagen-mediated delivery method was superior to the method with the liposome (79).

Very promising results have been obtained using cyclodextrin-containing polymers (CDP) (80). These polymers are also polycationic and contain imidazole endgroups to assist in the intracellular trafficking (81). The polymers self-assemble with siRNA to form colloidal particles of about 50 nm. For systemic delivery, Hu-Lieskovan et al. stabilized these particles using an adamantane-PEG conjugate (AD-PEG), based on inclusion complex formation between adamantane and cyclodextrins. Some of the PEG chains contained transferrin as a tumor-targeting ligand. Reduction of the EWS-FLI1 protein, involved in tumorigenesis of Ewing's family of tumors (EFT), was demonstrated after tail-vein injection of EWS-FLI1 siRNA loaded CDP nanoparticles on two consecutive days, while long-term delivery (twice weekly for 4 weeks) almost completely inhibited growth of metastasized EFT (82). These systems may have a broad applicability in cancer therapy, targeting different genes and/or tumor types. For instance, administration of these particles carrying siRNA against ribonucleotide reductase subunit M2 (RRM2) also led to growth inhibition of subcutaneous Neuro2A tumors (83). Similar nanoparticles are currently also being evaluated for the treatment of cancer, as the first systemically delivered clinically applied siRNA complexes (CALAA-01 from Calando Pharmaceuticals).

Dynamic PolyConjugates are interesting systems for the delivery of siRNA to hepatocytes (84). In these systems, siRNA, the shielding agent PEG and the hepatocyte targeting agent *N*-acetylgalactosamine (NAG) are reversibly attached to an endosomolytic polymer composed of butyl and amino vinyl ethers (PBAVE). In the low

pH environment of the endosomes, the system disassembles, unmasking the polymer's amine groups and activating its endosomolytic properties. Using this technology, effective silencing of apolipoprotein B (apoB) in the liver was demonstrated after i.v. injection, which resulted in a significant reduction in serum cholesterol. It is anticipated that incorporation of other ligands into the system enables targeting to other tissues or cell types.

Recently, systemic delivery of siRNA has also been shown for orally administered formulations. Oral delivery of siRNA to macrophages in mice was accomplished using β1,3-D-glucan-encapsulated siRNA particles (GeRPs), which were targeted to macrophages in the gut-associated lymphatic tissue (GALT). Interestingly, downregulation of the target gene was also discovered in macrophages from the spleen, lung and liver, which is believed to be the result of GALT macrophages trafficking away from the gut and infiltrating other tissues that are part of the reticuloendothelial system. Silencing of Map4k4 in macrophages protected mice from lipopolysaccharide-induced lethality by inhibition of TNF-α and interleukin-1β production. GeRP-mediated delivery of siRNA may therefore be a new strategy to attenuate inflammatory responses in human disease (85).

Other polymers that have been used for systemic delivery of siRNA include branched histidine-lysine-rich (HK) polymers, that, in complex with Raf-1 siRNA, could inhibit growth of MDA-MB-435 xenografts (86) and star vectors (SVs), which are four-branched diblock copolymers containing an inner domain of cationic (poly(*N,N*-dimethylaminopropylacrylamide) chains and an outer domain of nonionic (poly(*N,N*-dimethylacrylamide) chains. Intravenous injection of SV/siRNA complexes into mice led to effective gene silencing in the liver and lung, offering another possible therapeutic approach in gene therapy (87).

Safety issues

Polymers are attractive for the delivery of siRNA, however their application is hampered by safety concerns. These concerns can be classified into acute and delayed effects and are dependent on polymer characteristics such as molecular weight and degree of branching of the polymer and size and zeta potential of the polyplexes. Acute effects usually result from the cationic nature of the polymer. For PEI, it has been shown that systemic administration leads to interaction of the cationic polymers with negatively charged serum proteins and red blood cells, causing aggregation, blockade of capillaries and adherence to cell surfaces (88). These effects can be prevented by shielding of the charge using non-ionic polymers like PEG. More important and difficult to overcome are delayed effects that occur after polyplexes have left the circulation and are taken up by cells. After decomplexation, the polymers can interact with cellular components and interfere with normal cellular processes. They can cause cell shrinkage, formation of

vacuoles in the cytoplasm and reduce number of mitoses (89). Biodegradable polymers are currently being investigated to circumvent these cellular effects.
Furthermore, their structure, originally designed to interact with siRNA may equally efficiently interact with DNA, mRNA and small regulatory RNAs (in addition to proteins and lipids). Recent toxicogenomics studies have shown that polymers can also elicit wide ranging gene changes in cells (90). Altered gene expression profiles for cells that had taken up polymers have already been shown for several polymers including PEI (90), PPI (91), PEG (92) and polyamidoamine (PAMAM)-based dendrimers (93). These off-target effects can affect the safety of the formulation. Furthermore, if polymers alter the expression of genes that are targeted by the siRNA, this may affect the silencing efficiency. Hollins et al. showed that two commercially available PAMAM dendrimers, Polyfect (PF) and Superfect (SF), had opposing effects on epidermal growth factor receptor (EGFR) expression (93). Treatment of cells with PF resulted in EGFR mRNA downregulation of 50%, whereas treatment with SF led to a 2-fold increase. As a result, markedly more potent silencing was achieved with an anti-EGFR siRNA delivered with PF than with SF, which was not due to variations in cell uptake of siRNA. In general, understanding the effects of a delivery system in terms of its impact on gene silencing activity and specificity is essential for optimal use.
Furthermore, despite possibilities of acute and delayed effects, a study by Heidel et al., tested the safety of polyplexes in non-human primates (94). For their targeted CDP polyplexes (see previous section) they performed an extensive toxicity study in cynomolgus monkeys. Escalating intravenous doses of 3, 9 and 27 mg siRNA/kg were administered and blood samples were drawn for analyses of complete blood counts, serum chemistry, coagulation parameters, complement factors, antibodies, cytokines and pharmacokinetics. In general, the particles were well tolerated at doses of 3 and 9 mg siRNA/kg. At 27 mg siRNA/kg, an increase in blood urea nitrogen and creatinine was found, indicative of renal dysfunction. Furthermore, a mild increase in aspartate transaminase and alanine amino transferase was measured suggesting that there may have been some adverse effect on the liver. Coagulation parameters were not affected, whereas modest elevation of specific cytokines was observed, suggesting limited immunostimulation. Overall, no clinical signs of toxicity attributable to the treatments were observed. These data suggest that multiple, systemic doses of the nanoparticles can safely be administered to non-human primates.

CONCLUSIONS

RNAi has become one of the most promising approaches in the development of therapeutics for a wide range of diseases, but successes in (pre-) clinical studies are still limited due to inefficient delivery of siRNA to the target site. Polymer-based systems are

promising delivery systems that can be chemically tailored for efficient siRNA protection and intracellular delivery. Although results of studies using polymers are increasingly encouraging, more insight into the mechanism of cellular delivery and pharmacokinetics of these systems will help to develop novel and better delivery systems. Moving towards clinical use, safety issues will become of higher importance, which will broaden the need for safe and biodegradable systems that are well characterized for their off-target effects.

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Chapter 4

A method for quantifying cellular uptake of fluorescently labeled siRNA

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ABSTRACT

Efficient intracellular delivery of siRNA is a significant hurdle to its therapeutic success. For biological studies on the efficiency of carrier-mediated uptake of siRNA, quantitative determination of the amount of internalized siRNA is required. In this study, when the apparent uptake of fluorescently labeled siRNA, formulated in different lipo- and polyplexes, was examined using different techniques, major differences were observed. Additional experiments showed that these differences could be explained by quenching phenomena that were dependent on interactions between siRNA and carrier and their intracellular environment. Differences in fluorescent signal of complexed siRNA due to quenching could be overcome by measuring the fluorescent signal after lysing the transfected cells in lysis buffer that contained 2% SDS to dissociate siRNA from the complexes. This method offers a simple approach for quantifying cellular uptake of siRNA, which might help in the development of more efficient delivery systems.

INTRODUCTION

RNA interference (RNAi) is a technique to induce sequence-specific gene silencing (1, 2). Besides its use in loss-of-function studies, RNAi has emerged as a promising new therapeutic strategy, inhibiting expression of disease-related genes (3, 4). Gene silencing is mediated by short interfering RNA (siRNA), which are double stranded RNA molecules (19-27 nt) that can bind to mRNA in the cytoplasm of cells via complementary base pairing and induce mRNA degradation. However, since siRNA molecules are highly negatively charged and are therefore not readily taken up by cells, much effort has been put in the development of safe and efficient siRNA delivery systems (5). These systems have to complex siRNA and ensure cellular uptake, endosomal escape and unpacking of the complex in the cytosol. For optimization of delivery systems, all of the above mentioned steps in the overall process of delivery have to be thoroughly investigated separately.

To be able to compare different delivery systems on their ability to ensure cellular internalization, techniques to quantitatively determine the amount of siRNA inside cells after transfection are required. Quantitative information can be obtained using radiolabeled siRNA, which can be detected in cellular preparations by liquid hybridization (6) or liquid scintillation counting (7). However, working with radioactive probes requires trained personnel, laboratory licensing and special waste management. The use of Q-PCR has also been described for the quantification of siRNA present inside cells (8, 9), but requires expensive material and extensive optimization. Fluorescently labeled siRNAs on the other hand are commercially available, cheap, easy to detect and therefore frequently used to determine and/or visualize cellular uptake. However, we have observed major differences in the apparent uptake of siRNA formulated in several lipo- and polyplexes, dependent on the technique used to determine the fluorescent signal. In this study, these differences were further explored, and we offer concrete recommendations for the detection of fluorescent siRNA in cells for quantitative or comparative studies.

MATERIALS & METHODS

Materials

Lipofectamine2000 (LF) was purchased from Invitrogen (Breda, The Netherlands), branched PEI (bPEI) (M_w ~ 25 kDa) from Sigma-Aldrich (Zwijndrecht, The Netherlands) and FuGENE HD Transfection reagent from Roche Diagnostics GmbH (Mannheim, Germany). Reducible poly(amido amine)s based on cystaminebisacrylamide and 4-amino-1-butanol (pABOL) or cystaminebisacrylamide, 4-amino-1-butanol and 1,2-diaminobutane (pABOL-EDA) were synthesized by Michael addition as previously

described (10). The cationic methacrylamide-based polymer pHPMA-MPPM was synthesized as previously described (11). Polymers were dissolved in 20 mM Hepes buffer containing 5% glucose (HBG). siRNAs (5'-UGCGCUACGAUCGACGAUGTT-3' and 5'-CAUCGUCGAUCGUAGCGCATT-3') were chemically synthesized and supplied by Eurogentec (Maastricht, The Netherlands). The 5'-end of the sense strand was modified with Alexa488 dye. For cell studies, the human head and neck squamous cell carcinoma cell line UM-SCC-14C (14C) was used, kindly provided by Prof. Dr. G.A.M.S. van Dongen (Department of Otolaryngology, Head and Neck Surgery, VU Medical Center, Amsterdam, The Netherlands). Cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM) containing 3.7 g/liter sodium bicarbonate, 4.5 g/liter L-glucose, 2 mM L-glutamine, 5% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B.

Preparation of polyplexes and lipoplexes

Branched PEI and pHPMA-MPPM polyplexes were prepared at N/P ratio 8, unless stated differently. Polyplexes of pABOL and pABOL-EDA were prepared at w/w ratio 24 (polymer/siRNA). Lipoplexes with LF and FuGENE were prepared at their optimal ratio, according to the manufacturer's protocol. Appropriate amounts of polymer or lipid and siRNA were diluted in HBG or PBS and mixed by adding the polymer/lipid solution to the siRNA solution (volume ratio 4:1). Mixtures were vortexed for 5 s and incubated for 30 minutes. For determination of fluorescent signal of siRNA inside lipo- or polyplexes, 20 μl of complex solution (containing 20 pmol siRNA) was transferred to a black 96-well plate. After addition of 180 μl of the appropriate buffer (with or without 2% SDS), fluorescence was determined on a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany), equipped with FITC filters. Fluorescent values were corrected for background signal from the buffer and expressed as relative to fluorescent signal of free siRNA.

Agarose gel retardation

For gel retardation studies, complexes were prepared in a total volume of 30 μl containing 70 pmol siRNA. After incubation, samples were transferred to new eppendorf tubes. To one half, SDS was added to a final concentration of 2%. An equal volume of H_2O was added to the other half. Finally, 6x loading dye (Fermentas, St. Leon-Rot, Germany) was added and samples were loaded on a 4% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was performed at 80 V for 60 min, after which pictures of the gel were made under UV-illumination.

Fluorescent signal in- and outside cells

$8x10^3$ 14C cells per well were seeded in black 96-well plates and incubated for 24 hours. Then, medium was replaced with serum- and antibiotics-free medium and cells were treated with LF lipoplexes, at a final concentration of 66 nM siRNA. After 4 hours, PBS, Triton in PBS (final conc. 1%) or Triton/SDS in PBS (final conc. 1% and 2% respectively) was added to the wells. Fluorescent signals were measured on a Mithras LB 940, corrected for background signals and expressed as relative to the signal of free siRNA.

Uptake studies

Flow cytometry – $4x10^4$ 14C cells per well were seeded in 24-well plates and incubated for 24 hours. Then, medium was replaced with serum- and antibiotics-free medium and cells were treated with lipo- or polyplexes, at a final concentration of 66 nM siRNA. After 4 hours, cells were washed twice with PBS, trypsinized, transferred to a 96-well plate and spun down (5 min, 300 *g*, 4°C). Cells were washed again, spun down, and resuspended in FACS buffer (0.3% BSA in PBS). Mean fluorescence intensity of the cells was measured on a FACSCanto (Becton & Dickinson, Mountain View, CA, USA).

Determination of fluorescence in cell lysates – $1.6x10^5$ 14C cells per well were seeded in 6-well plates and incubated for 24 hours. Then, medium was replaced with serum- and antibiotics-free medium and cells were treated with lipo- or polyplexes, at a final concentration of 66 nM siRNA. After 4 hours, cells were washed twice with PBS and lysed in 400 µl lysis buffer (1% Triton X-100, 2% SDS in PBS) on ice for 30 minutes. Lysates were transferred to eppendorf tubes and centrifuged (15 min, 14000 *g*, 4°C) to remove cell debris. Then, 200 µl of the supernatant was transferred to a black 96-well plate for measuring the fluorescence on a Mithras LB 940. 50 µl was used to determine the protein content using the Micro BCA™ protein assay (Pierce, Rockford, USA), according to the protocol of the manufacturer. For determination of the mean fluorescence intensity, fluorescent signals were corrected for the amount of protein in the samples.

Fluorescent microscopy - $8x10^3$ 14C cells per well were seeded in 12-well plates on coverslips and incubated for 24 hours. Then, medium was replaced with serum- and antibiotics-free medium and cells were treated with lipo- or polyplexes, at a final concentration of 66 nM siRNA. After 4 hours, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes. After washing, nuclei were stained with DAPI for 5 minutes. Finally, slides were mounted with Fluorsave Reagent (Calbiochem, San Diego, CA, USA), dried and imaged using a Nikon TE2000-U microscope (Nikon Benelux, Bruxelles, Belgium).

RESULTS & DISCUSSION

Efficient cellular uptake represents one of the major hurdles towards the therapeutic use of siRNA (5). To compare different delivery systems on their ability to deliver siRNAs to 14C cells, Alexa488-labeled siRNA was formulated in different lipo- and polyplexes. Cellular uptake was determined in three different ways (Figure 1). First, cells were analyzed by flow cytometry and the mean fluorescent intensity of the cells was used as a

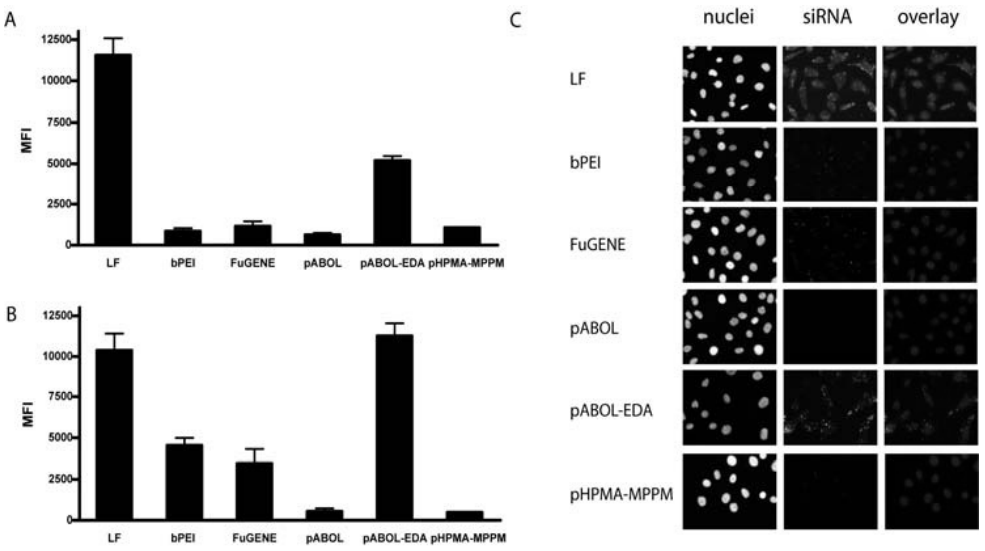

Figure 1. Three different ways to determine cellular uptake of Alexa488-siRNA formulated in different lipo- and polyplexes by 14C cells. (A) Mean fluorescence intensity (MFI) of treated cells as determined by flow cytometry. (B) MFI of cells as determined by measuring the fluorescent signal in cell lysates. Fluorescent values were corrected for the protein content. (C) Microscopy images of treated cells. Nuclei are stained blue (DAPI) and the siRNA appears as green (Alexa488).

measure for the amount of internalized siRNA (Figure 1A). This approach is widely used to compare uptake of siRNA formulated in different carriers (12-14). MFI of cells transfected with LF was significantly higher than of cells transfected with pABOL-EDA. MFI of cells transfected with bPEI was much lower, comparable to FuGENE, pABOL and pHPMA-MPPM. Interestingly, when MFI values were determined in cells that were lysed in PBS containing 1% Triton X-100 and 2% SDS, different results were obtained. MFI for pABOL-EDA was found to be similar to LF, and values for bPEI and FuGENE exceeded

the values for pABOL and pHPMA-MPPM by several orders of magnitude (Figure 1B). Findings for uptake as evaluated by microscopy were in general consistent with data obtained by flow cytometry analysis, showing high fluorescent intensity of cells transfected with LF and pABOL-EDA and no or very faint fluorescence for the other lipo- and polyplexes (Figure 1C).

The differences in apparent uptake of siRNA between different detection methods might be due to quenching effects, as quenching of the fluorescent signal could lead to lower MFI values. To further investigate this hypothesis, we prepared lipo- and polyplexes and determined the fluorescent signal of Alexa488 siRNA after complex formation. Indeed, the fluorescent signal of siRNA formulated in lipo- and polyplexes was found to be much lower than that of unformulated (free) siRNA (Figure 2A, left). This phenomenon is not limited to Alexa488 fluorescent labels, as quenching of cy3-siRNA that was formulated in nanoparticles has also been described (15). Furthermore, signals also differed among different poly- and lipoplexes. This could be explained by differences in interaction

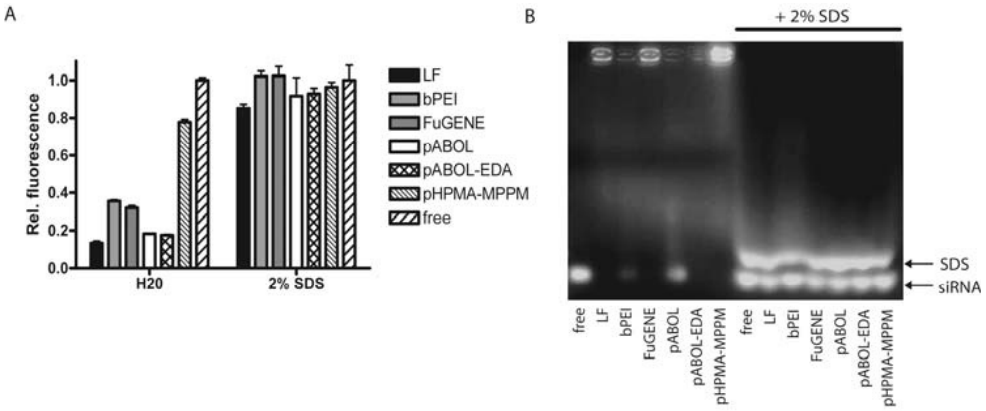

Figure 2. Fluorescent signal of siRNA is quenched inside lipo- and polyplexes and can be restored by addition of 2% SDS. (A) Relative fluorescent signal of siRNA after complexation with different lipo- and polyplexes as compared to free siRNA. For all complexes, addition of 2% SDS to the solution completely restores the fluorescent signal. (B) Agarose gel retardation of different siRNA lipo- and polyplexes, in the presence (right) or absence (left) of 2% SDS.

between carrier and siRNA, as tight compaction of fluorescent labels increases quenching due to their close spatial proximity (16). As determination of fluorescence by flow cytometry or microscopy does not take these differences into account, the extent of uptake of fluorescent siRNA that is tightly complexed by its carrier might be underestimated using one of these techniques. On the contrary, fluorescence measurements in cell lysates after lysing in buffer containing 2% SDS is not hampered

by these differences, as addition of 2% SDS causes dissociation of all lipo- and polyplexes, releasing siRNA, as shown by agarose gel retardation (Figure 2B), resulting in complete recovery of the fluorescent signal of siRNA (Figure 2A, right). The differences between apparent uptake as determined using flow cytometry or in cell lysates could be explained by increased condensation or slower release of siRNA from bPEI, FuGENE and ABOL-EDA complexes compared to LF, ABOL and pHPMA-MPPM. Indeed, homogeneous green cell staining was found for LF-transfected cells, indicating that siRNA was released from the complex, while for pABOL-EDA transfected cells a more punctuated pattern was found, which could be interpreted as siRNA associated with the carrier (13) (Figure 1C).

Differences in quenching degree are not only found among different systems, but also for a single carrier formulated in different buffers or at different carrier:siRNA ratios. When the same lipo- and polyplexes were prepared in PBS instead of HGB, not only the absolute fluorescent signals changed, but also the relative values for siRNA in the different carriers. These differences disappeared after addition of 2% SDS, which restored fluorescent values for all complexes to that of free siRNA (Figure 3A).

For optimization of transfection efficiency, it is important to be able to compare uptake of siRNA formulated in complexes at different carrier-to-siRNA ratios (17, 18). However, as shown in Figure 3B for bPEI, the relative fluorescence of siRNA formulated at different N/P ratios varied. For higher N/P ratios, increased quenching was found, probably due to increased condensation as described above. If uptake of these particles would be determined using flow cytometry or microscopy, the uptake of complexes at higher N/P ratios could be underestimated. Again, when fluorescence was determined after addition of 2% SDS, values were found to be almost equal to those for free siRNA.

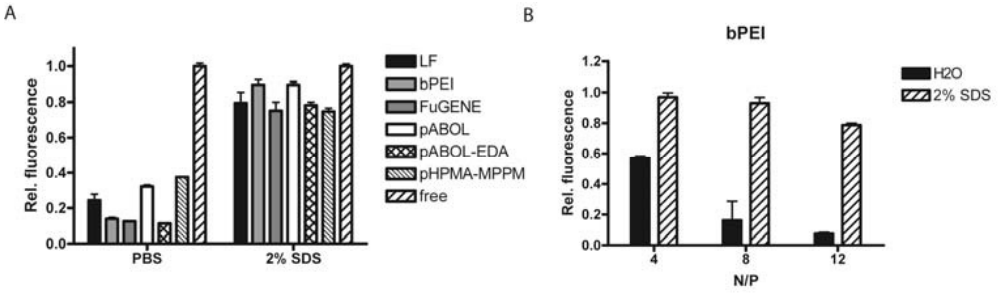

Figure 3. Degree of quenching of fluorescent siRNA in lipo- and polyplexes depends on medium and N/P ratio. (A) Relative fluorescent signal of siRNA after complexation with different lipo- and polyplexes in PBS as compared to free siRNA. Addition of 2% SDS to the solution completely restores the fluorescent signal. (B) Relative fluorescent signal of siRNA after complexation with bPEI at different N/P ratios as compared to free siRNA. For all ratios, addition of 2% SDS to the solution restores the fluorescent signal.

Quenching of fluorescent signals has also been shown to occur inside cells due to differences in pH (13) or intracellular location (19). When cells were incubated with LF lipoplexes and the fluorescent signals from the wells were determined, signals were found to be only a fraction of the signal from free siRNA. When cells were lysed with 1% Triton X-100 before measurement, the signals were increased, indicating that at least part of signal might have been lower due to cellular uptake of lipoplexes. However, a large part of the signal was still quenched after cell lysis, as 1% Triton X-100 had no effect on the fluorescent signal inside particles (data not shown). Only a lysis buffer that consisted of 1% Triton X-100 and 2% SDS caused almost total recovery of the fluorescent signal (Figure 4).

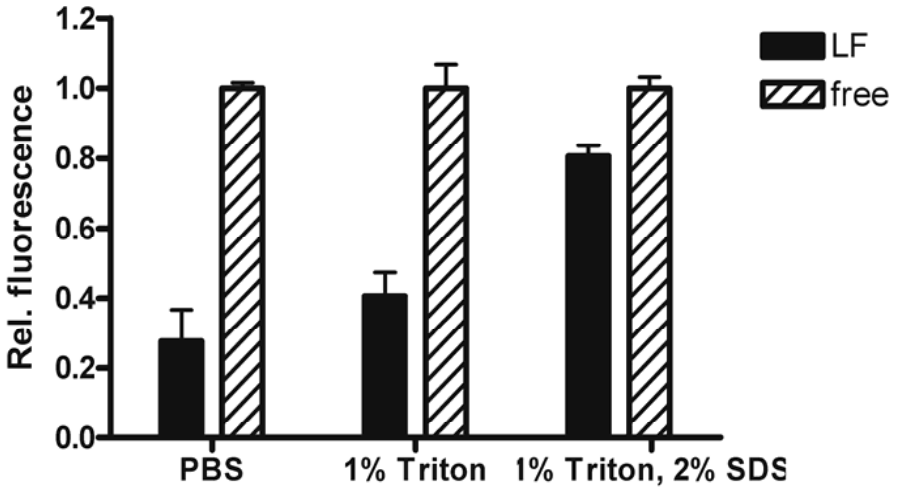

Figure 4. Fluorescent signal of siRNA is quenched inside LF-complexes on the in- and outside of 14C cells. Signal is almost completely restored by treating cells with lysis buffer containing 2% SDS.

In general, to be able to quantitatively compare uptake of siRNA formulated in different carriers or at different N/P ratios, fluorescence should be measured after cell lysis and dissociation of lipo- and polyplexes, to ensure that quenching of the fluorescent signal from the siRNA does not occur. For carriers investigated in this study, a simple lysis buffer consisting of 1% Triton X-100 and 2% SDS could be used, however for other carriers a different lysis buffer might be more appropriate, depending on the nature and strength of interactions between siRNA and carrier (15, 20, 21). Still, determination of uptake using flow cytometry or microscopy could provide additional information, as differences between results obtained using these techniques and fluorescent

measurements in cell lysates can often be explained by tight condensation of the siRNA, which has shown to have a negative influence on gene silencing efficiency (18).

CONCLUSIONS

For the development of efficient delivery systems for siRNA, straightforward quantitative comparison of uptake of fluorescently labeled siRNA into cells leads to better understanding of the process of lipo- and polyplex uptake and the physico-chemical characteristics that influence it. However, due to quenching effects, determination of uptake using flow cytometry or microscopy is not quantitative and might lead to incorrect conclusions, as the intensity of the fluorescent signal depends on the tightness of complexation or time course of release of free siRNA into the cytosol. Measuring of the fluorescent intensity after cell lysis and dissociation of the complexes is not hampered by these issues and offers a simple approach for quantitative and comparative studies of siRNA uptake.

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Chapter 5

Disulfide-based poly(amido amine)s for siRNA delivery. Effects of structure on siRNA complexation, cellular uptake, gene silencing and toxicity

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ABSTRACT

Purpose: RNA interference (RNAi) is a process by which small interfering RNAs (siRNA) induce sequence-specific gene silencing. Therefore, siRNA is an emerging promise as a novel therapeutic. In order to realize the high expectations for therapeutic applications, efficient delivery systems for siRNA are necessary.
Methods: In this study a new series of biodegradable poly(amido amine)s with disulfide linkages in the backbone was synthesized out of N,N'-cystaminebisacrylamide (CBA), 4-amino-1-butanol (ABOL) and ethylene diamine (EDA). Effects of different percentages of butanolic side chains and protonatable fragments in the main chain on siRNA complexation, cellular uptake, gene silencing and toxicity were investigated.
Results: Incorporation of EDA in the polymer resulted in increased siRNA condensation. Efficient siRNA condensation was shown to be necessary for cellular uptake, however excess of polymer decreased siRNA uptake for polymers with high amounts of EDA. Silencing efficiency did not correlate with uptake, since silencing increased with increasing w/w ratio for all polymers. More than 80% knockdown was found for polyplexes formed with polymers containing 25% or 50% EDA, which was combined with low cytotoxicity.
Conclusions: Poly(amido amine)s with minor fractions of protonatable fragments in the main chain are promising carriers for delivery of siRNA.

INTRODUCTION

RNA interference (RNAi) is an evolutionary conserved process for post-transcriptional silencing (1, 2). Introduction of short interfering RNA (siRNA) molecules into cells can effectuate RNAi (3). The technique is currently widely used as a tool in functional genomics, but it also holds great promise as a therapeutic strategy, by suppressing the expression of disease-related genes (4, 5). However, since siRNA molecules are relatively large and highly negatively charged they are not readily taken up by cells. Therefore, the development of efficient and safe delivery systems that deliver siRNA to its site of action, i.e. the cytoplasm, is essential for therapeutic activity. These systems should fulfill several requirements, including the ability to protect siRNA during the extracellular delivery stage, enhance cellular association and uptake, trigger endosomal escape and release siRNA in the cytoplasm. For clinical use, they should furthermore be safe and biodegradable. Current methods for siRNA delivery include viral vectors, peptides (6), cationic lipids (7), liposomes (8) and cationic polymers such as poly(ethylenimine) (PEI) (9) and poly(L-lysine) (PLL) (10). Although these approaches have all shown specific advantages and disadvantages, the attractiveness of polymers lies in the possibility to be specifically tailored for different applications. At the same time, the major drawback of the currently used polymers is their high toxicity, which is most likely caused by their poor biodegradability.

Recently, a new class of biodegradable cationic polymers based on poly(amido amine)s with disulfide linkages in the backbone (SS-PAA polymers) has been developed (11). These polymers self assemble with plasmid DNA into nano-sized polyplexes and display efficient gene transfer properties. Due to the difference in redox potential between the oxidizing extracellular space and the reducing intracellular space the disulfide bonds are stable outside the cell, but are rapidly cleaved in the cytoplasm. Introduction of disulfide linkages in the polymer chain has already been shown to result in increased transfection efficiency, due to an increased release of DNA from the complexes, and decreased toxicity for this class of polymers (12). From the SS-PAAs tested by Lin et al., the copolymer of N,N'-cystaminebisacrylamide (CBA) and 4-amino-1-butanol (ABOL) showed the best balance between DNA transfection efficiency and toxicity (12). The origin of the positive effect of the butanolic side chains of this polymer on transfection efficiency still has to be elucidated, however it has also been found for a similar class of polymers (13).

Although delivery of DNA and siRNA faces the same challenges, their molecular topography is different, which highlights different requirements to their respective delivery systems (14). In this study, CBA was copolymerized besides ABOL with 1,2-diaminoethane (EDA) as the amine monomer to introduce more positive charges in the polymer (Figure 1). This is expected to increase the electrostatic interactions between

polymer and siRNA, resulting in improved complexation. Copolymers containing different percentages of butanolic side chains and aminoethyl fragments in the main chain were synthesized and evaluated for their potential use for siRNA delivery by comparing their siRNA condensation properties, cellular uptake, gene silencing efficiency and toxicity.

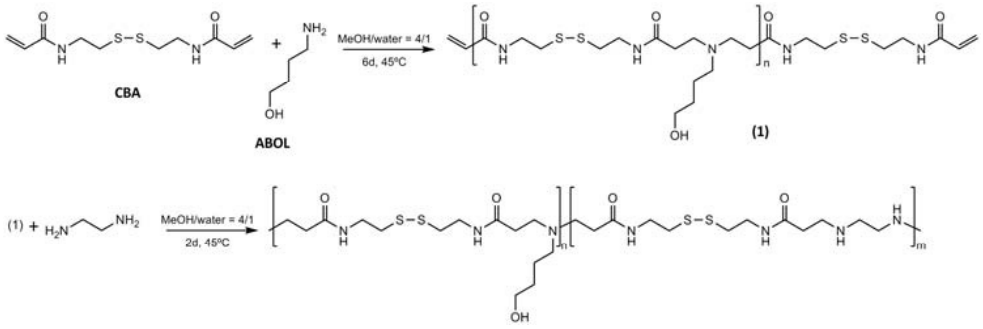

Figure 1. Synthesis of poly(CBA-ABOL/EDA) copolymers by prepolymerization of CBA with ABOL and subsequently with EDA monomers.

MATERIALS & METHODS

siRNAs

siRNAs were chemically synthesized and supplied by Eurogentec (Maastricht, The Netherlands). The sequence of epidermal growth factor receptor (EGFR) siRNA was 5'-GUU-UGC-CAA-GGC-ACG-AGU-AdTdT-3'; 3'-dTdTC-AAA-CGG-UUC-CGU-GCU-CAU-5'. For uptake studies, the 5'-end of the sense strand was modified with Alexa 488 dye. Negative control siRNA (Eurogentec) was used for gel retardation assays, size and zeta potential measurements and cell viability experiments.

Polymer synthesis and characterization

The poly(amidoamine) copolymers were synthesized by Michael addition polymerization reactions as previously described (12, 15). However, the amine monomers 4-amino-1-butanol (ABOL) and 1,2-diaminoethane (EDA) were added sequentially to the reaction mixture. This is necessary since EDA contains two primary amino groups and therefore, in principle, can react four times with acrylamide moieties in the Michael addition, which results in branching and formation of networks. By first allowing all ABOL monomer to react with the acrylamide groups to form linear prepolymer chains with acrylamide terminal groups, and subsequently add an equimolar amount of EDA, branching is

prevented as much as possible, since the primary amines in EDA have a higher reactivity than the newly formed secondary amines in the polymer chain. Therefore, *N,N'*-cystaminebisacrylamide (CBA) and ABOL were first prepolymerized in methanol / water (4/1) at 45ºC (Figure 1). After six days of polymerization EDA was added and after two days the polymerization was terminated by addition of an excess of EDA. The product was purified by ultrafiltration (MWCO 1000, pH 5), filtered through a 0.45 μm filter and recovered by lyophilization, and analyzed by ^{1}H NMR and GPC.

Polyplex formation

For polyplexes at different polymer/siRNA (w/w) ratios, siRNA and the appropriate amount of polymer solution were each diluted in HBG (Hepes-Glucose buffer: 20 mM Hepes, pH 7.4, 5% glucose). Next, the polymer solution was added to the siRNA solution in an Eppendorf tube, at a volume ratio of polymer solution to siRNA solution of 4:1. The mixture was vortexed for 5 s and incubated for 30 min at room temperature before use. Lipoplexes with Lipofectamine2000 (Invitrogen, Breda, The Netherlands), used as a control, were prepared at the optimal Lipofectamine2000:siRNA ratio, according to the manufacturer's protocol.

Gel retardation assay

Polyplexes, containing 125 pmol siRNA, were prepared in 50 μl HBG. Polyplexes were incubated for 1 h at 37°C in the presence or absence of 5 mM glutathione. Next, 4 μl 6x loading dye (Fermentas) was added to 20 μl of polyplex solution and samples were loaded on a 4% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was performed at 90 V for 30 min, after which pictures of the gel were made under UV-illumination.

Size and ζ- potential measurements

Polyplexes, containing 200 pmol siRNA, were prepared in 500 μl HBG. Hydrodynamic diameters were determined using dynamic light scattering on an ALV CGS-3 system (Malvern Instruments Ltd., Worcestershire, United Kingdom). For ζ- potential measurements, polyplexes were 1:1 diluted in HBG and measured by laser Doppler electrophoresis using a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom).

Cell culture

The human head and neck squamous cell carcinoma (HNSCC) cell line UM-SCC-14C, abbreviated as 14C, was kindly provided by Prof. Dr. G.A.M.S. van Dongen (Department of Otolaryngology, Head and Neck Surgery, VU Medical Center, Amsterdam, The Netherlands). Cells were cultivated at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's

medium (DMEM) containing 3.7 g/liter sodium bicarbonate, 4.5 g/liter L-glucose and 2 mM L-glutamine, supplemented with 5% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B.

Cellular uptake measurements

Qualitative uptake - 14C cells ($8x10^3$ per well) were seeded in 12-well plates on coverslips, 48 h before transfection. Medium was replaced with serum-free medium and cells were treated with 20 µl of different polyplexes, at a final concentration of 66 nM fluorescently labeled siRNA. After 4 h, cells were washed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After fixation, cells were washed twice with PBS, mounted on glass cover slides using FluorSave (Calbiochem, San Diego, CA, USA) and allowed to air dry overnight. Cells were then imaged by a Nikon TE2000-U fluorescent microscope, equipped with a GFP-B filter (Nikon Benelux, Brussels, Belgium).

Quantitative uptake - Quantitative uptake of siRNA was determined as previously described, with minor modifications (16). 14C cells ($1.6x10^5$ cells per well) were seeded in 6-well plates, 24 h before transfection. Medium was replaced with serum-free medium and cells were treated with 400 µl of different polyplexes, at a final concentration of 66 nM fluorescently labeled siRNA. After 4 h, cells were put on ice and washed with PBS, 1M NaCl and again PBS to remove all non-internalized complexes (Figure S1). 400 µl lysis buffer (2% SDS, 1% Triton X-100 in PBS) was added to solubilize cells and dissociate polyplexes. Cells were lysed for 1 h on ice, after which the lysate was centrifuged (15 min, 14000 *g*, 4°C) to remove cell debris. 200 µl of the supernatant was transferred to a black 96-well plate to measure fluorescence on a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany). A calibration curve of fluorescent siRNA in lysis buffer was used to calculate the amount of internalized siRNA. Aliquots (25 µL) of the supernatants were used to determine the cellular protein content using the Micro BCA™ protein assay (Pierce, Rockford, USA), according to the instructions of the supplier. The amount of internalized siRNA was normalized to the amount of protein and uptake was calculated as percentage of total siRNA input.

Gene silencing experiments

Silencing experiments were performed as previously described (17). 14C cells ($4x10^4$ cells per well) were seeded in 24-well plates, 24 h before transfection. Medium was replaced with serum-free medium and cells were treated with 100 µl of different polyplexes, at a final concentration of 66 nM anti-EGFR siRNA. After 4 h, medium was replaced with complete medium and cells were incubated for another 48 h. Cells were trypsinized, washed with cold PBA buffer (0.3% BSA, 0.03% sodium azide in PBS) and incubated with a specific FITC-labeled anti-EGFR monoclonal antibody (Santa Cruz

Biotechnology Inc., Santa Cruz, CA, USA) in 150 µl PBA buffer for 30 min at 4°C. After that, cells were washed twice with cold PBA buffer and resuspended in 300 µl PBA buffer. Measurements were taken on a FACSCalibur (Becton & Dickinson, Mountain View, CA, USA) and analyzed using WinMDI 2.9 (©1993-2000 Joseph Trotter). In a density plot representing forward scatter and sideward scatter, whole cells were gated out and at least 5000 cells were counted for each sample. The mean fluorescent intensity (MFI) served as a measure of the amount of EGFR present on the cells.

Cell proliferation assay

14C cells ($8x10^3$ cells per well) were seeded in 96-well plates, 24 h before transfection. Medium was replaced with serum-free medium and cells were treated with 20 µl of different polyplexes, at a final concentration of 66 nM siRNA. After 4 h, medium was replaced with complete medium and cells were incubated for 24 h. BrdU reagent was added to a final concentration of 10 µM and cells were cultured for another 24 h. Colorimetric BrdU cell proliferation assay (Roche Applied Science, Penzberg, Germany) was performed according to the supplier's instructions.

Statistical analysis

Results were analyzed using ANOVA with Bonferroni post-tests to assess statistical significance.

RESULTS & DISCUSSION

In this study a new series of poly(amido amine) copolymers was explored, based on the copolymer of N,N'-cystaminebisacrylamide (CBA) and 4-amino-1-butanol (ABOL), which has recently been shown to efficiently deliver plasmid DNA to COS-7 cells (12). This polymer was tailored for siRNA delivery by copolymerizing 1,2-diaminoethane (EDA) besides ABOL to increase the density of amine groups in the main chain of the polymer. Since previous studies have already shown that small changes in the structure of the polymer can affect the delivery efficiency (11, 18), copolymers containing different percentages of ABOL and EDA fragments were synthesized. The characteristics of the final polymers, poly(CBA-ABOL/EDA), are shown in Table I. The compositions of the polymers were in accordance with the aimed composition, molecular weights ranged between 1.6 and 11.7 kDa and polydispersity indices were comparable for all polymers.

Table I

Characteristics of synthesized CBA-ABOL/EDA copolymers

	Feed composition[1]	*Obtained composition*[2]	*Yield (%)*[3]	M_w *(kg/mol)*[4]	*PDI*
ABOL/EDA	100/0		27	4.1	1.4
	75/25	80/20	50	11.7	2.4
	50/50	56/44	57	7.2	1.9
	25/75	30/70	53	3.1	1.6
	0/100		45	1.6	1.6

[1] Stoichiometric ratio. [2] Determined by ^{1}H NMR. [3] after ultrafiltration. [4] Determined by GPC.

Polyplex formation

Formation of polyplexes was confirmed by gel retardation. When siRNA is efficiently bound to its carrier and subsequently shielded from the environment, migration into the gel is completely retarded. Polyplexes were formed at w/w ratios 0 (naked siRNA) to 48. Results are shown in Figure 2, left panel. Poly(CBA-ABOL) could not efficiently encapsulate siRNA. Only at the highest w/w ratio tested, siRNA was completely bound to its carrier (Figure 2A, left panel). As hypothesized, addition of EDA to the final copolymer resulted in increased complexation of siRNA at lower w/w ratios. Complete retardation was achieved at w/w ratios 12 and higher for polymers with 25% EDA and 6 and higher for polymers with 50% and 75% EDA respectively (Figure 2B, C, D, left panel). Poly(CBA-EDA) showed efficient complexation at w/w ratio 3 and higher (Figure 2E, left panel). This increased condensation ability with increasing percentage of EDA in the main chain of the polymer can be explained by the increased charge density of the polymer, since, after incorporation of EDA, increased complexation of siRNA was observed at lower w/w ratios.

Although for cellular uptake and protection against nucleases siRNA has to be complexed, for efficient gene silencing siRNA should be completely released from the complex inside the cell (15, 19). One of the micro-environmental features which has been exploited for improving the delivery of nucleic acids, is the redox potential gradient that exists between the extra- and intracellular environment (20). A high concentration of glutathione (± 5 mM (21)) inside cells causes rapid cleavage of disulfide bonds. The disulfide bridges in the poly(CBA-ABOL/EDA) copolymers are also expected to be cleaved in a reductive environment. To investigate whether this reductive degradation also results in siRNA release from the complex, polyplexes were incubated for 1 h at 37°C in the presence of 5 mM glutathione, mimicking the intracellular environment, and subsequently subjected to electrophoresis as described. As shown in Figure 2, right panel, cleavage of disulfide bridges resulted in release of siRNA, for all polymers, even at the

highest w/w ratios. These results suggest that siRNA is effectively condensated (Figure 2, left panel), but is released from the complex after successful cellular uptake.

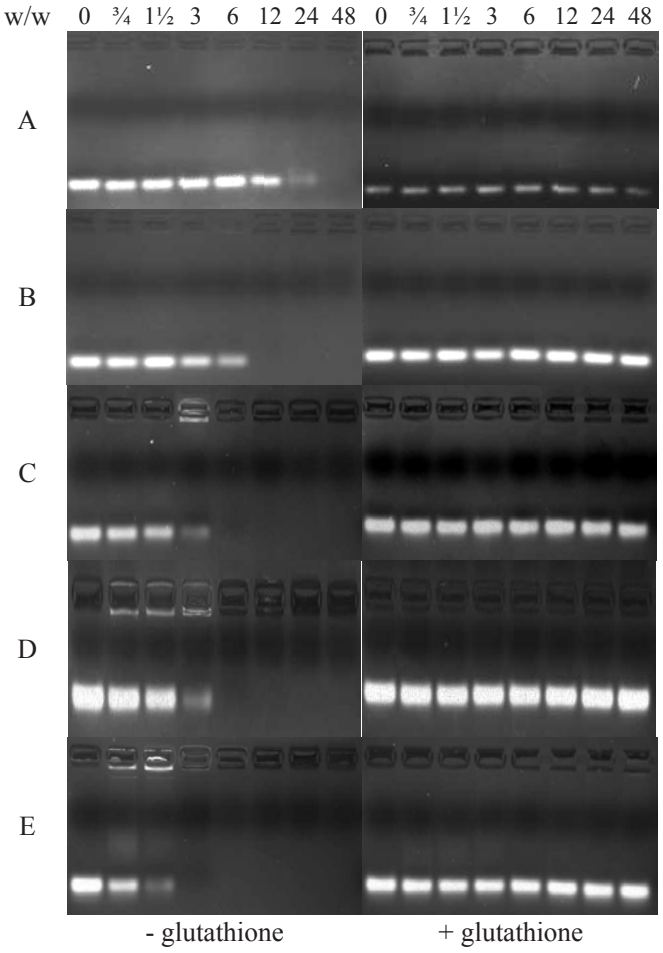

Figure 2. Gel retardation assay of siRNA polyplexes at w/w ratio from 0 (siRNA only) to 48, after 1 hr incubation in the presence (right) or absence (left) of 5 mM glutathione. Polyplexes were built with polymers containing different percentages of ABOL/EDA (100/0 (A), 75/25 (B), 50/50 (C), 25/75 (D) and 0/100 (E)).

Polyplexes were further characterized by size and ζ-potential measurements. Polyplexes were formed at w/w 3, 6, 12, 24 and 48. Size and surface charge measurements are presented in Figure 3A and 3B. For all polymers, increasing the w/w ratio resulted in smaller particles with higher ζ-potential. Poly(CBA-ABOL) was only able to condense

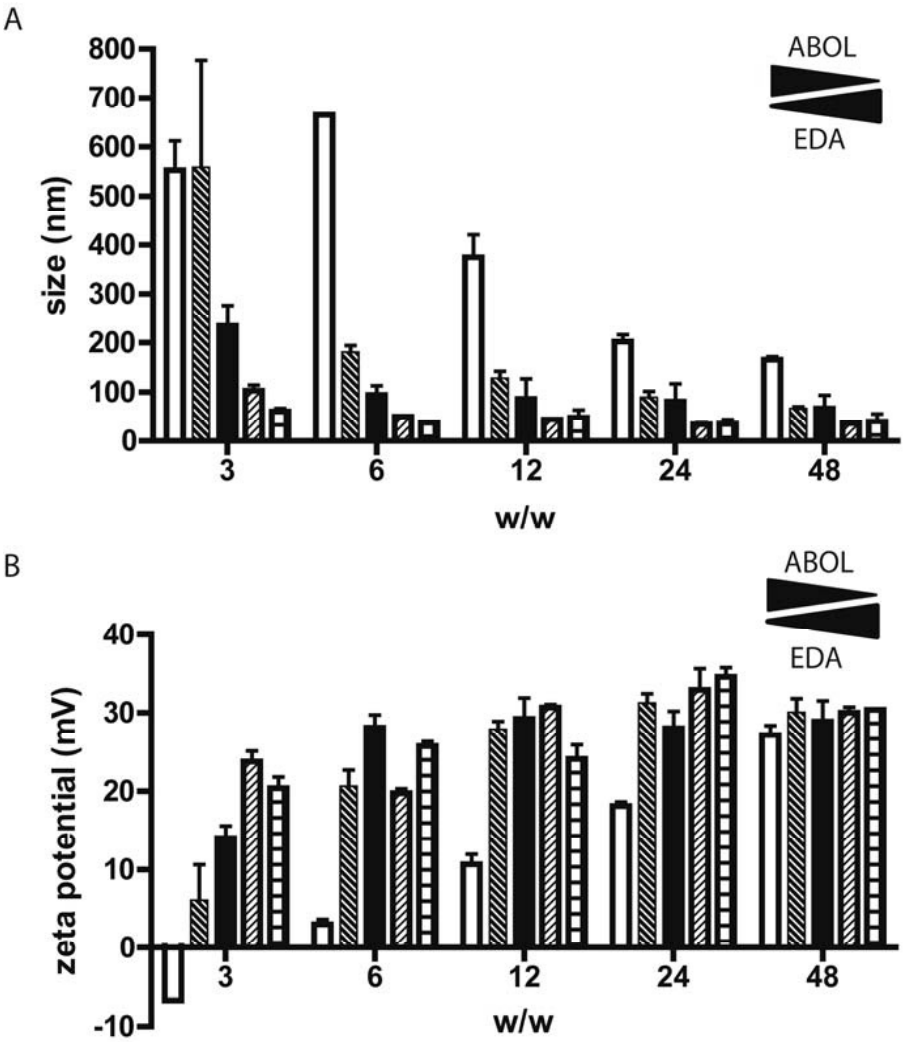

Figure 3. Particle size (A) and ζ-potential (B) of poly(CBA-ABOL/EDA) / siRNA polyplexes prepared in 20 mM Hepes + 5% glucose, at w/w ratio of 3, 6, 12, 24 and 48. Polyplexes were built with polymers containing different percentages of ABOL/EDA (100/0 (white bars), 75/25 (black, hatched bars), 50/50 (black bars), 25/75 (white, hatched bars) and 0/100 (white, blocked bars)). Particle size and ζ-potential were determined by dynamic light scattering and laser Doppler electrophoresis. Results are reported as mean ± SD for 2-3 individual measurements.

siRNA into small particles at the higher w/w ratios 24 and 48. Polymers with 25% or 50% EDA instead of ABOL more efficiently condensed siRNA, resulting in particles smaller than 200 nm for w/w ratio 6 and higher. For the copolymers containing 75% or 100% EDA, all polyplexes formed were smaller than 150 nm. Surface charge

measurements followed the same trend as the size measurements. For all w/w ratios, ζ-potential of the complexes increased with the amount of EDA in the polymer. For copolymers containing 75% or 100% EDA, the ζ-potential of the complexes were all almost equally positively charged to polyplexes at w/w ratio 3, with ζ-potentials between 20 and 30 mV. Interestingly, for polymers with 50%, 75% and 100% EDA, from w/w ratio 12, 3 and 3 respectively, addition of more polymer to the final complex did not result in smaller particles or higher surface charges. This suggests that from these w/w ratios an excess of 'free' polymer was added, which had no effect on the physicochemical properties of the final complex.

Cellular uptake

Cellular uptake is one of the key steps in the process of siRNA delivery. To assess the ability of the various polymers to facilitate siRNA internalization, fluorescently labeled siRNA was used to form polyplexes. First, uptake was studied using fluorescent microscopy. The medium was replaced with serum-free medium during the time of transfection (4h), as binding of serum proteins to the polyplexes makes it more difficult to exclusively determine the effect of polymer composition on its activity. The fluorescent signal in the cells treated with poly(CBA-ABOL) complexes was very weak. Only at the highest w/w ratios, few cells displayed a faint green staining (Figure 4A). This indicates that efficient siRNA condensation is a prerequisite for cellular uptake. Introduction of 25% EDA in the final polymer resulted in increased uptake of polyplexes at w/w ratio 12, 24 and 48. Cells displayed a mixture of discrete green spots and a weaker diffuse green signal, interpreted as siRNA associated with or released from the carrier respectively (19). At the lowest w/w ratios, fluorescent intensity was still very low (Figure 4B). Cells transfected with polyplexes formed with polymers with 50% or 75% EDA instead of ABOL showed uptake for all w/w ratios, however at the highest w/w ratios fluorescence was less intense (Figure 4C, D). For poly(CBA-EDA), fluorescence could only be detected in cells treated with polyplexes at the lowest w/w ratios (Figure 4E). After uptake of Lipofectamine2000 complexes, used as a control, cells displayed a similar fluorescent signal (Figure 4F). For all polymers that showed uptake also a diffuse fluorescent signal appeared, suggesting effective siRNA release from the complexes. Improved cytosolic availability of siRNA may increase the chance of effective gene silencing (22).

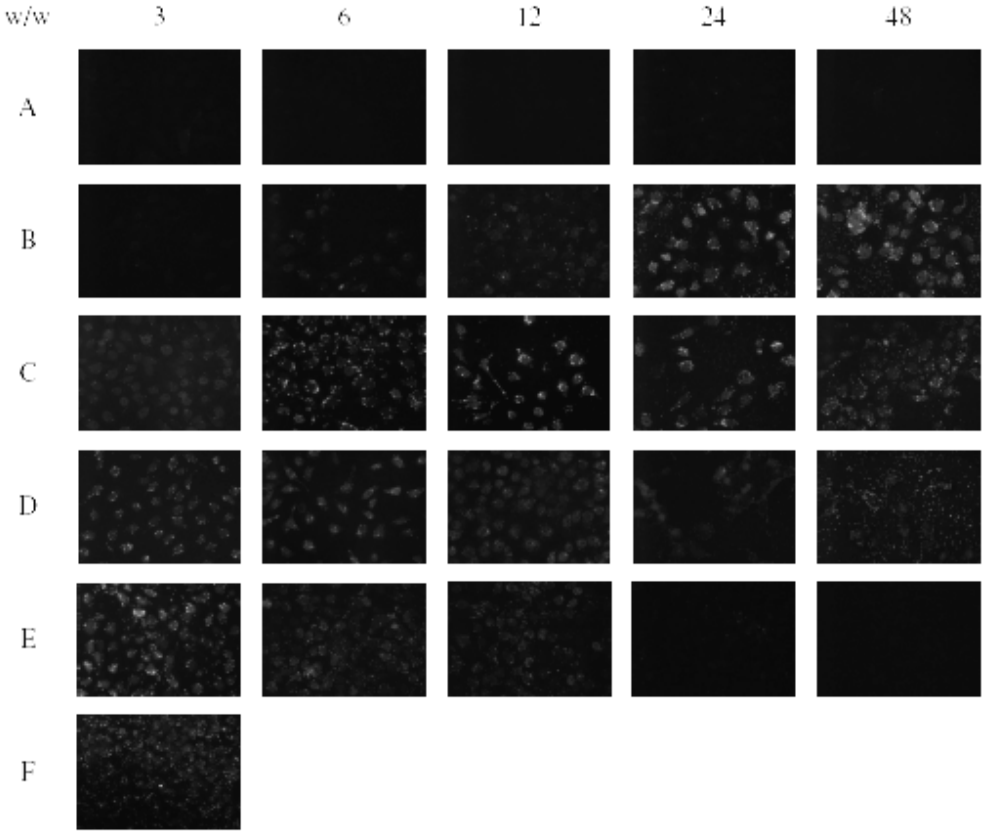

Figure 4. Fluorescent microscopy pictures of 14C cells after treatment with polyplexes at w/w ratio of 3, 6, 12, 24 and 48. Polyplexes were built with polymers containing different percentages of ABOL/EDA (100/0 (A), 75/25 (B), 50/50 (C), 25/75 (D) and 0/100 (E)). Lipofectamine2000 (F) was used as a control.

Although microscopy images suggested efficient release from the carrier after uptake and showed major differences in uptake efficiency, the ability of different polymers to enhance siRNA internalization was also quantitatively determined. For absolute quantification, fluorescence was measured in cell lysates, since fluorescent signals located in endosomes often cannot be detected due to quenching effects. Furthermore, since complexation of siRNA with polymer also results in loss of fluorescent signal, 2% sodium dodecyl sulfate (SDS) was added to the lysis buffer, resulting in polyplex dissociation and total signal restoration (data not shown). Results are shown in Figure 5. Consistent with the fluorescent microscopy data, poly(CBA-ABOL) polyplexes showed very little uptake, even at w/w ratio 48 (0.3% of input). Higher percentages of EDA in the polymer resulted in much more effective uptake. For polyplexes formed with the

copolymer containing 75% ABOL and 25% EDA, uptake increased with increasing w/w ratio to a maximum of 14%. Surprisingly, for polymers containing 50%, 75% or 100% EDA, polyplexes were most efficiently taken up at lower w/w ratios, with uptake maxima of 15%, 10% and 7% at w/w 12, 3 and 3 respectively.

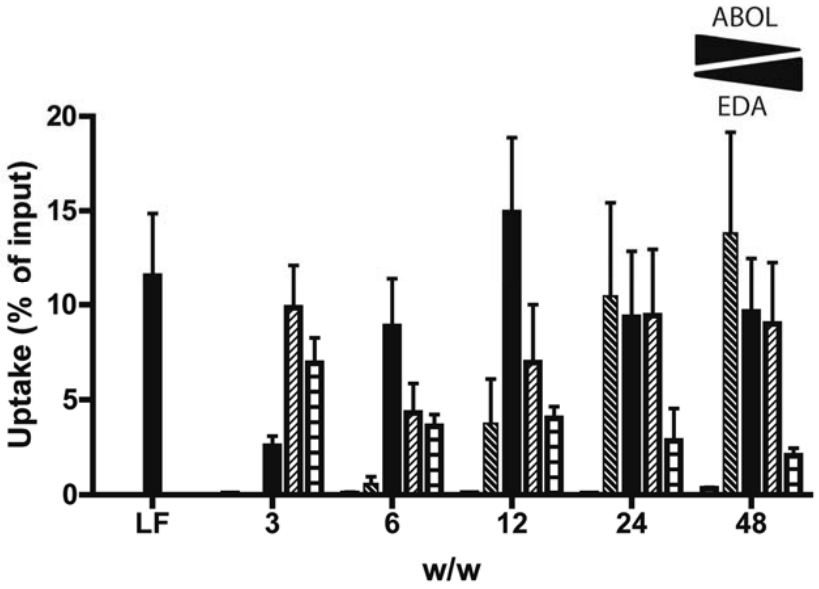

Figure 5. siRNA uptake in 14C cells. Cells were treated with polyplexes at w/w ratio of 3, 6, 12, 24 and 48. Polyplexes were built with polymers containing different percentages of ABOL/EDA (100/0 (white bars), 75/25 (black, hatched bars), 50/50 (black bars), 25/75 (white, hatched bars) and 0/100 (white, blocked bars)). Lipofectamine2000 (LF) was used as a control. Uptake was reported as mean uptake ± SD for n=3-4.

As mentioned above, for these polymers complete condensation of siRNA was established at these w/w ratios. Thus, at higher w/w ratios, free polymer may have hampered siRNA uptake and this effect is expected to be pronounced in case of polymers with high charge densities, as was also found in the fluorescent microscopy pictures (Figure 4 C, D and E). This is most likely due to shielding of the heparin surface proteoglycans present on cells by free polymer, resulting in reduced polyplex association, as has been reported earlier for PEI-polyplexes (23). Alternatively, as colloidal stability of the polyplexes increases with increasing w/w ratio, decreased aggregation of the polyplexes could result in lower uptake. However, such decreased aggregation at higher w/w ratio was not observed under the conditions used in our transfection studies, as is shown for polyplexes prepared with polymers containing ABOL/EDA ratios of 75/25 and

0/100 (Supplementary Figure 2). In general, uptake maxima were comparable to uptake of Lipofectamine2000 lipoplexes (12%).

Gene silencing and toxicity

Silencing experiments were performed on a 14C carcinoma cell line, using siRNA against the epidermal growth factor receptor (EGFR) as an example of a therapeutic target. EGFR is overexpressed in many tumors and is known to play a role in cell proliferation, migration, angiogenesis development and inhibition of apoptosis (24). Polyplexes were formed at w/w 3, 6, 12, 24 and 48 for all polymers. As shown in Figure 6, for all polymers increasing the w/w ratio resulted in decreased expression of EGFR after transfection. This effect was specifically caused by siRNA against EGFR, as treatment with the same polyplexes containing negative control siRNA had no effect on EGFR expression, as shown for w/w ratio 48 (Figure 6B).

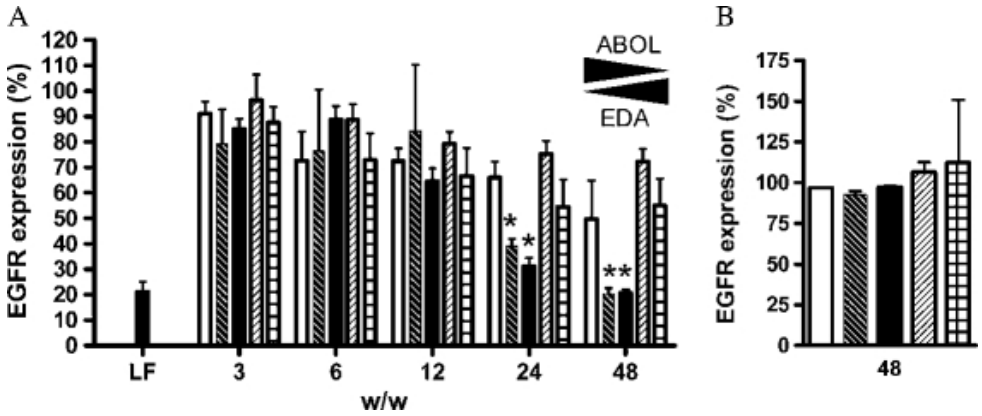

Figure 6. Gene silencing of EGFR in 14C cells at a final concentration of 66 nM siRNA. Cells were treated with polyplexes at w/w ratio of 3, 6, 12, 24 and 48 using EGFR siRNA (A) or w/w 48 using negative control siRNA (B). Polyplexes were built with polymers containing different percentages of ABOL/EDA (100/0 (white bars), 75/25 (black, hatched bars), 50/50 (black bars), 25/75 (white, hatched bars) and 0/100 (white, blocked bars)). Lipofectamine2000 (LF) was used as a control. EGFR expression was analyzed by flow cytometry and reported as mean expression ± SD for n=2-6. Statistical significant differences of polymers containing ABOL/EDA percentages of 75/25 and 50/50 from other polymers are denoted by *.

The same amount of polymer without siRNA also did not decrease EGFR expression (Supplementary Figure 3). In contrast to our data on siRNA uptake (Figure 4 and 5), for all polymers maximal silencing efficiency was obtained at the highest w/w ratio tested. Therefore, uptake of polyplexes does not seem to be the only limiting step in the

transfection process. Another major hurdle for transfection is known to be endosomal escape. These poly(amido amine)s contain amino groups with different pK_a's, introducing high buffering capacity for membrane disruption, either via the proton sponge effect (25) or conformational changes induced by pH changes (26). Our results indicate that a certain threshold amount of polymer is necessary to achieve efficient endosomal escape. For example, while for the polymer containing 50% ABOL and 50% EDA siRNA uptake was higher at w/w ratio 12 than at w/w ratio 48, silencing efficiency was much less (approximately 30% versus 80%, respectively). Likely, the addition of extra polymer contributed to the overall buffer capacity of the complexes inside endosomes. Our data suggest that free polymer, as in the case of polyplexes formed with polymers containing more than 50% EDA at the highest w/w ratios, can also have a positive effect on endosomal escape and subsequent gene silencing. Similar results have been shown for PEI (27).

Interestingly, striking differences in maximum silencing efficiency were found between the different copolymers. Polyplexes formed with polymers containing 25% or 50% EDA were able to induce >80% EGFR silencing, which was even slightly better than for Lipofectamine2000 lipoplexes, the current standard for *in vitro* siRNA delivery. Only moderate silencing (approximately 40%) was found for siRNA formulated with the other polymers. The same trend was observed, when cells were transfected in the presence of serum. Only treatment with polyplexes prepared at w/w ratio 48 with polymers containing 25% or 50% EDA resulted in EGFR knockdown, although at higher siRNA concentration (200 nM) (Supplementary Figure 4).

For poly(CBA-ABOL) and poly(CBA-EDA) complexes, only moderate silencing in the absence of serum can be attributed to their poor uptake (Figure 4 and 5). On the contrary, the amount of siRNA that was taken up after complexation with the polymer containing 75% EDA was almost equal to the amount taken up after polyplex formation with the polymers with 25% or 50% EDA (Figure 5). Of the three polymers, the first has the highest amount of amine groups and therefore the strongest interaction with siRNA. It is likely that, despite reduction of disulfide bonds in the cytoplasm, the inability to completely release siRNA limited silencing efficiency for this polymer. Likewise, of the three bioreducible polymers that were tested by Christensen et al., the polymer with the highest charge densities of amines produced the lowest overall gene expression (15).

Relative cell viability was evaluated by determining the ability of cells to proliferate after treatment. As show in Figure 7, polyplexes from polymers with 0% or 25% EDA were non-toxic, even at the highest w/w ratios. For the polymer with 50% EDA, polyplexes reduced cell proliferation to approximately 50% at w/w ratio 48. Polymers with 75% or 100% EDA were the most toxic, with respectively 40% and 10% viability after treatment with polyplexes at w/w ratio 24 and only a few percent viable cells at w/w 48. In general, polyplexes were more toxic at increasing w/w ratio. Furthermore, increasing the amount

of EDA in the polymer also resulted in decreased cell viability. Toxicity has previously been shown to be related to charge density of the polymer (28). Comparing these results with the results from the physicochemical characterization of the complexes, toxicity might be primarily caused by addition of an excess of polymer, an observation that is confirmed by other studies (23). Interestingly, while often transfection efficiency seems to be correlated to toxicity, in our study the most toxic polymers were shown to be the least effective.

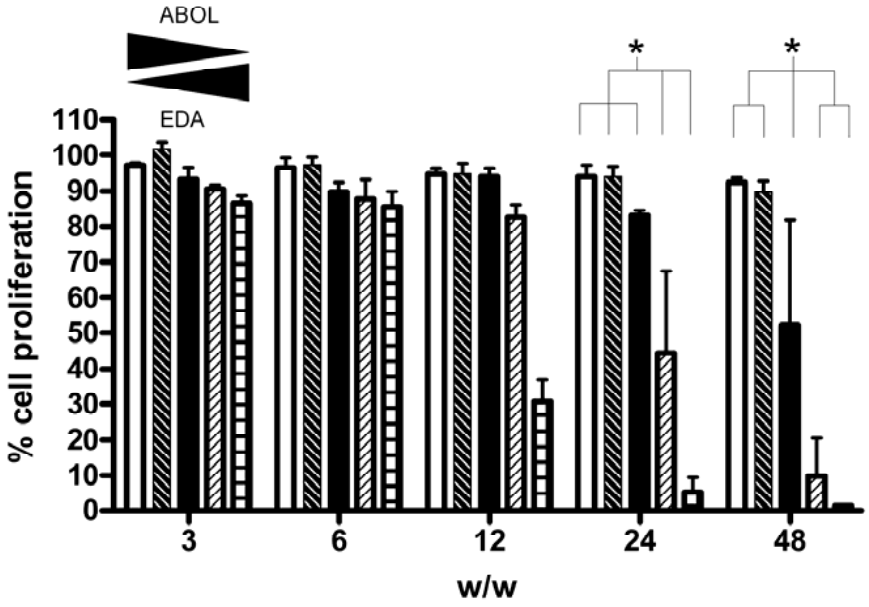

Figure 7. Percentage of cell proliferation of 14C cells after treatment with poly(CBA-ABOL/EDA) / siRNA polyplexes, evaluated using the BrdU proliferation assay. Polyplexes were built with polymers containing different percentages of ABOL/EDA (100/0 (white bars), 75/25 (black, hatched bars), 50/50 (black bars), 25/75 (white, hatched bars) and 0/100 (blocked bars)). Results are reported as mean ± SD for n=3. Statistically significant differences are denoted by * ($p<0.05$).

In the discussion of the results, differences in molecular weight between the polymers were not taken into account, although molecular weight has shown to be of influence on siRNA condensation and toxicity (29). However, the obtained results can be explained by differences in chemical composition between the polymers, as complexation efficiency and toxicity correlate with composition of the polymers, but not with molecular weight.

CONCLUSIONS

In this study, several bioreducible poly(amido amine) copolymers were tested for their ability to deliver siRNA to a carcinoma cell line and induce gene silencing. These copolymers were based on a previously described polymer poly(CBA-ABOL), which has shown to be able to efficiently transfect pDNA. Copolymers with different percentages of EDA and ABOL were synthesized and the interaction with relatively small siRNA molecules was studied. As predicted, poly(CBA-ABOL) was not able to completely condense siRNA into nano-sized polyplexes, resulting in minor cellular uptake and gene silencing. Addition of 25% or 50% EDA instead of ABOL to the polymer resulted in polyplexes from respective w/w ratios of 12 and higher and 6 and higher. These polyplexes were efficiently taken up by 14C cells. Transfection resulted in effective gene silencing at higher w/w ratios with only minor toxicity. Polymers with high percentages of EDA (75% or 100%) formed small polyplexes from the lowest w/w ratios. Efficient uptake was only found at low w/w ratios, indicating that an excess of polymer hindered uptake at higher w/w ratios. However, uptake of polyplexes with low w/w ratios did not result in effective gene silencing, probably due to lack of sufficient polymer to induce endosomal escape.

Taken together, we have shown that introduction of extra amine groups in the CBA-ABOL polymer increased siRNA condensation resulting in complete shielding and smaller, more positively charged polyplexes at lower w/w ratios. Only siRNA that was effectively complexed was taken up by cells; however excess of polymer hindered this process. At the same time, sufficient amount of polymer was necessary for endosomal escape. Our data indicate that CBA-ABOL polymers with a minor amount of EDA are most feasible for siRNA delivery. In the future, the potential of these polymers for their use for *in vivo* siRNA delivery will be evaluated.

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Supporting information

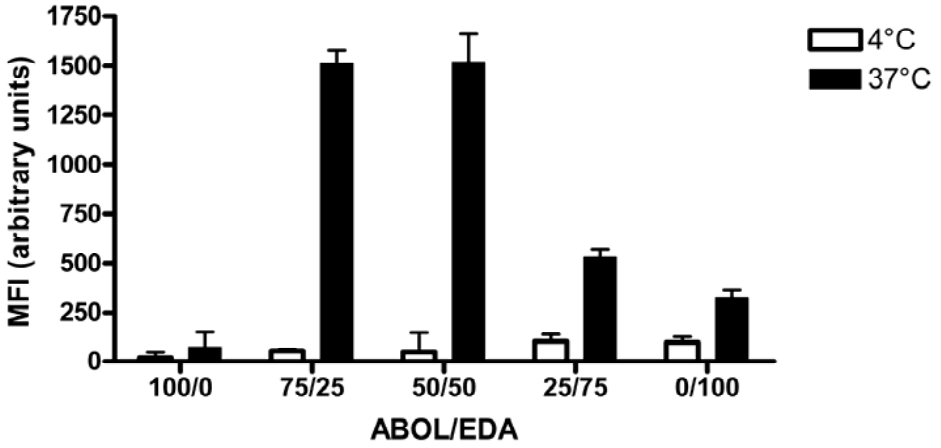

Figure S1. siRNA uptake in 14C cells at 4°C (white bars) or 37°C (black bars). Cells were treated with polyplexes at w/w ratio 48, using polymers containing different percentages of ABOL/EDA. Uptake was reported as mean fluorescent intensity of treated cells (MFI) ± SD for n=3. Results show that at 4°C (no endocytosis, only binding), almost no fluorescence was observed, indicating efficient removal of surface-bound polyplexes from cells.

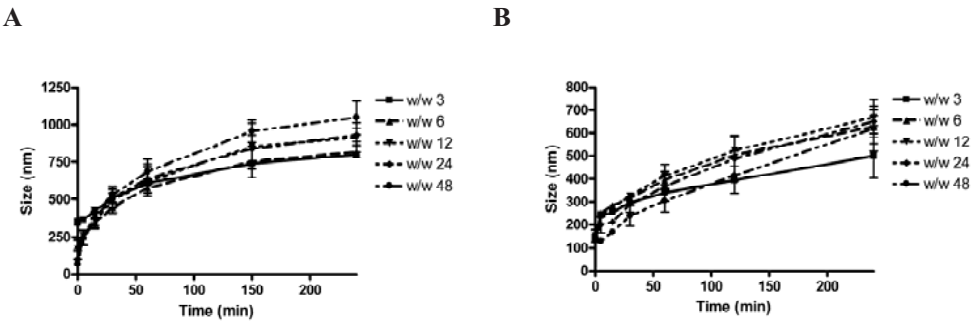

Figure S2. Particle size of poly(CBA-ABOL/EDA) / siRNA polyplexes, prepared at w/w ratio 48, under concentrations and conditions used in transfection studies over time. Polyplexes were prepared with polymers containing percentages of ABOL/EDA of 75/25 (A) and 0/100 (B) in 400 µl 20 mM Hepes + 5% glucose, after which 2 ml serum-free medium was added to a final siRNA concentration of 66 nM (t=0). Particle sizes were determined over time by dynamic light scattering and reported as mean ± SD for 2 individual measurements.

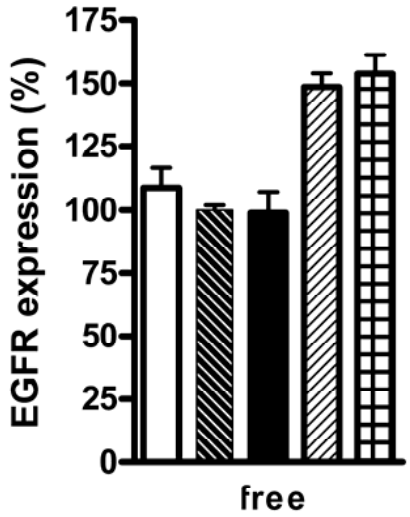

Figure S3. EGFR expression of 14C cells treated with free polymer at polymer concentration equivalent to the concentration cells were subjected to when treated with polyplexes prepared at w/w ratio 48. Cells were treated with polymers containing different percentages of ABOL/EDA (100/0 (white bars), 75/25 (black, hatched bars), 50/50 (black bars), 25/75 (white, hatched bars) and 0/100 (white, blocked bars). EGFR expression was analyzed by flow cytometry and reported as mean expression ± SD for n=3.

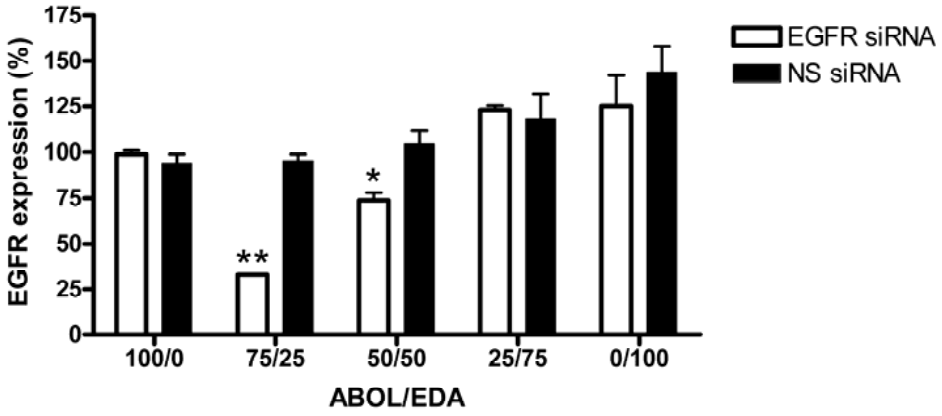

Figure S4. Gene silencing of EGFR in 14C cells in the presence of serum at a final concentration of 200 nM siRNA. Cells were treated with polyplexes at w/w ratio of 48 using EGFR siRNA (white bars) or negative control (NS) siRNA (black bars). EGFR expression was analyzed by flow cytometry and reported as mean expression ± SD for n=3. For statistical analysis of differences between EGFR expression of cells treated with EGFR or negative control siRNA an unpaired Student't t-test was used (*$p<0.01$, **$p<0.001$).

Chapter 6

Physicochemical and biological evaluation of siRNA polyplexes based on PEGylated poly(amido amine)s

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ABSTRACT

Purpose: The use of RNA interference as novel therapeutic strategy is hampered by inefficient delivery of its mediator, siRNA, to target cells. Cationic polymers have been thoroughly investigated for this purpose, but often display unfavorable characteristics for systemic administration, such as interactions with serum and/or toxicity.
Methods: In this study, the synthesis of a new PEGylated polymer is reported, based on biodegradable poly(amido amine)s with disulfide linkages in the backbone. Various amounts of PEGylated polymers were mixed with their unPEGylated counterparts prior to polyplex formation to alter the PEG content in the final complex.
Results: PEGylation effectively decreased polyplex surface charge, salt- or serum-induced aggregation and interaction with erythrocytes. However, increasing the amount of PEG in the formulation also reduced its stability against heparin displacement, cellular uptake and subsequent silencing efficiency. Nevertheless, for polyplexes with high PEG content, significant gene silencing efficacy was found, which was combined with almost no toxicity. Conclusions: These results indicate that PEGylated poly(amido amine)s are promising carriers for systemic siRNA delivery *in vivo*.

INTRODUCTION

RNA interference (RNAi) using small interfering RNAs (siRNAs) has emerged as a powerful tool for knockdown of genes and holds great potential as a novel therapeutic strategy for a broad range of diseases (1, 2). The primary limitation to clinical success, however, is the lack of efficient delivery systems that can deliver siRNA to the target cell population. siRNAs do not readily pass cellular membranes due to their negative charge and are rapidly degraded by serum nucleases.

Cationic polymers have widely been investigated for siRNA delivery due to their great flexibility, ease of manufacturing and modification possibilities (3). Typical examples include poly(ethyleneimine) (pEI) (4), poly(L-lysine) (pLL) (5), chitosan (6) and poly(2-(dimethyl-amino)ethylmethacrylate) (pDMAEMA) (7). Although significant progress has been made in improving these polymers for siRNA delivery, either low efficiency and/or high cytotoxicity remain to hinder their usefulness. Recently, we have described a new class of biodegradable polymers based on poly(amido amine)s with disulfide linkages in the backbone (SS-PAA) that were specifically tailored for delivery of siRNA (8, 9). These copolymers were composed of *N,N'*-cystaminebisacrylamide (CBA), 4-amino-1-butanol (ABOL) and ethylene diamine (EDA) (p(CBA-ABOL/EDA)) and were able to complex siRNA into positively charged polyplexes that were efficiently taken up by cells and induced target gene silencing. Moreover, this was combined with low cellular toxicity, which encouraged us to perform further functional studies.

For cancer therapy, in order to reach distant tumors or metastases, systemic administration of siRNA polyplexes is inevitable. Upon intravenous injection however, positively charged polyplexes might potentially interact aspecifically with serum proteins or erythrocytes and other blood cells, leading to formation of aggregates, which causes rapid clearance by the reticulo-endothelial systems (RES) and sometimes significant toxicity (10). The biocompatibility of polyplexes can be enhanced by conjugation of poly(ethylene glycol) (PEG) to the cationic polymer (PEGylation). Complexation of oligonucleotides with PEG-containing copolymers leads to the formation of particles with a core-shell structure, in which the cationic polymer packs the oligonucleotide within the particle core and the hydrophilic, non-charged PEG chains form a shell layer around it (11). In general, PEGylation of polyplexes results in a lower surface charge, reduced interaction with blood components, prolonged blood circulation and lower cytotoxicity (12, 13). On the other hand, steric shielding of the polyplex particles also leads to reduced cellular association and uptake, diminished endosomal escape properties, and inefficient siRNA release (14, 15). These contrasting effects associated with the use of PEG in oligonucleotide delivery is also referred to as the 'PEG dilemma' (16), and raise the need for strategies to fine-tune the PEG content in polyplexes to optimize their physicochemical and biological properties. Recently, Brumbach et al.

demonstrated the feasibility of altering and optimizing PEG content in polyplexes by formulating mixtures of a polycation and its corresponding PEGylated counterpart before complex formation, avoiding the need to synthesize multiple copolymers with varying degrees of PEGylation to identify optimal carrier candidates (17).
In this study we synthesized a new PEGylated polymer, p(CBA-ABOL/EDA/PEG), based on the successful p(CBA-ABOL/EDA) polymer and used mixtures of the PEGylated and unPEGylated polymer in order to vary the PEG content in the final polyplex. Polyplexes with different PEG content were compared to corresponding unPEGylated complexes regarding physicochemical characteristics, stability, cellular uptake, gene silencing activity and *in vitro* biocompatibility.

MATERIALS & METHODS

Materials

All chemicals, 4-amino-1-butanol (ABOL, Merck), ethylene diamine (EDA, Sigma-Aldrich), *N,N'*-cystaminebisacrylamide (CBA, Polysciences) and α-amino-ω-hydroxy poly(ethylene glycol) (PEG-NH_2, 3.000 g/mol, Iris Biotech GmbH) were purchased in the highest purity and used without further purification.

siRNAs

siRNAs were chemically synthesized and supplied by Eurogentec (Maastricht, The Netherlands). Sequence of siRNA against luciferase (siLuc) was 5'-GAU-UAU-GUC-CGG-UUA-UGU-AUU-3' (sense) and 5'-UAC-AUA-ACC-GGA-CAU-AAU-CUU-3' (antisense). For cellular uptake studies, Alexa-488-modified siRNA was used (dye was attached to the 5'-end of the sense strand). Universal negative control siRNA (siNS) was purchased from Eurogentec.

Polymer synthesis and characterization

Synthesis of p(CBA-ABOL/EDA) was performed as previously described (9). p(CBA-ABOL/EDA/PEG) was synthesized by Michael addition polymerization of ABOL, EDA and PEG-NH_2 with *N,N'*-cystaminebisacrylamide (CBA). Therefore, 424 mg (1.63 mmol) CBA, 98 mg (1.10 mmol) ABOL and 500 mg (0.17 mmol) PEG-NH_2 were dissolved in methanol/water (4/1 v/v) and were allowed to react at 45ºC in the dark in a nitrogen atmosphere. The reaction mixture became homogeneous within one hour. After six days of prepolymerization, 22 mg (0.37 mmol) EDA was added and the reaction was proceeded for another two days. Then the polymerization was terminated by addition of a 10% molar excess EDA, to consume remaining toxic acrylamide endgroups. After termination, the reaction mixture was diluted with hydrochloric acid (1M) and water, purified by ultrafiltration (MWCO 5000, pH5) and recovered as its HCl salt by

lyophilization. ^{1}H NMR (D_2O) δ (ppm) = 1.58 (m, 2H, ***CH$_2$***CH_2NR); 1.79 (m, 2H, ***CH$_2$***CH_2OH); 2.69 (t, 4H, NH***CH$_2$CH$_2$***NH); 2.77 (t, 2H, ***CH$_2$***CONH); 2.83 (t, 4H, ***CH$_2$***SS***CH$_2$***); 3.22 (t, 2H, CH_2***CH$_2$***NR); 3.32 (t, 2H, $COCH_2$***CH$_2$***NH); 3.44 (t, 4H, ***CH$_2$***NR***CH$_2$***); 3.51 (t, 4H, NH***CH$_2$***CH_2SSCH_2***CH$_2$***NH); 3.60 (t, 2H, ***CH$_2$***OH); 3.65-3.90 (m, 272H, O***CH$_2$CH$_2$***O). Polymers were characterized by ^{1}H NMR (D_2O), recorded on a Varian Innova spectrometer (300 MHz). Molecular weights were determined by GPC relative to PEG standards, using a GPCmax with an acetate buffer pH 4.5 containing 30% (v/v) methanol as eluent.

Polyplex formation

To prepare polyplexes at different polymer/siRNA (w/w) ratios, appropriate amounts of polymer and siRNA were each diluted in glucose-containing Hepes buffer (HBG: 20 mM Hepes, pH 7.4, 5 wt% glucose). Next, polymer solution was added to siRNA solution (4:1, v/v), followed by 5s vortexing and 30 min incubation at room temperature. To obtain polyplexes with different PEG content, expressed as the weight percentage of PEG to total polymer, polymer solutions of p(CBA-ABOL/EDA) and p(CBA-ABOL/EDA/PEG) were mixed prior to polyplex formation.

Polyplex characterization

For gel retardation assays, polyplexes were prepared in 50 µl HBG at a final siRNA concentration of 2.5 µM and incubated for 1 h at 37°C in the presence or absence of 5 mM glutathione. After addition of loading dye (Fermentas, St. Leon-Rot, Germany), samples were run on a 4% agarose gel containing 0.5 µg/ml ethidium bromide at 90V for 10 min. Hydrodynamic diameters and ζ-potentials were measured as previously described (8).

Complex aggregation in salt

Polyplexes were prepared in 400 µl HBG at a final siRNA concentration of 500 nM. Then, 2 ml PBS was added and hydrodynamic diameters were measured at indicated time points as described above.

Complex aggregation in serum

Polyplexes containing were prepared in 50 µl HBG at a final siRNA concentration of 15 µM. 180 µl fetal bovine serum (FBS) was added to 20 µl polyplex solution and samples were incubated for 10 min at 37°C. Aggregation in terms of turbidity increase of each sample was quantified by absorbance detection at 595 nm.

Stability of polyplexes against heparin

Polyplexes were prepared in 50 µl HBG at a final siRNA concentration of 2.5 µM and incubated for 30 min with heparin solutions of different concentrations, expressed as heparin/siRNA (w/w) ratio. After addition of loading dye, samples were run on a 4% agarose gel containing 0.5 µg/ml ethidium bromide at 90V for 10 min.

Erythrocyte aggregation and hemolysis

Erythrocytes were obtained from 200 µl whole murine blood by multiple centrifugation rounds (1000 *g*, 10 min, 4°C) followed by washing the pellet in PBS until the supernatant was clear. The final pellet was resuspended in 4 ml PBS. Subsequently, 160 µl erythrocyte suspension was added to 40 µl polyplex solution having a siRNA concentration of 4 µM and samples were incubated for 1h at 37°C. Triton X-100 (1%) in PBS (100% lysis) and HBG (0% lysis) were used as controls. After a final centrifugation step, 150 µl of the supernatant was analyzed for hemoglobin release by absorbance detection at 550 nm. The pellet was resuspended in 50 µl PBS for microscopic evaluation using a Nikon TE2000-U microscope.

Cell culture

H1299 (human lung cancer) cells stably expressing firefly luciferase were cultivated at 37°C and 5% CO_2 in RPMI 1640 (PAA laboratories GmbH, Pasching, Austria) supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B.

Cellular uptake

Quantification of siRNA uptake was performed as previously described, with minor modifications (18). H1299 cells were seeded in 6-well plates at a density of 1.6 x 10^5 cells per well, 24 hours before transfection. Cells were treated with 400 µl of different polyplexes containing Alexa-488 labeled siRNA at a final siRNA concentration of 125 nM in FBS-containing medium. After 4 hours, cells were washed twice with PBS to remove non-internalized polyplexes. 300 µl lysis buffer (2% SDS, 1% Triton X-100 in PBS) was added to lyse the cells and dissociate polyplexes. Cells were lysed for 1h on ice, after which the lysates were centrifuged (14000*g*, 15 min, 4°C) to remove cell debris. 200 µl supernatant was transferred to a black 96-well plate to measure fluorescence using a Fluostar OPTIMA microplate-based multi-detection reader (Isogen Life Science, Maarssen, The Netherlands). The mean fluorescence intensity was normalized to the amount of protein present in the sample, determined using the MicroBCA™ protein assay (Pierce, Rockford, USA).

For microscopy, cells were seeded in 12-well plates on coverslips, 24 hours before transfection. Cells were treated with 20 µl of different polyplexes containing Alexa-488

labeled siRNA at a final siRNA concentration of 125 nM. After 4 hours, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After fixation, slides were washed, counterstained with DAPI and mounted using Fluorsave (Calbiochem, San Diego, CA, USA). Cells were imaged using a Nikon TE2000-U fluorescent microscope (Nikon Benelux, Brussels, Belgium).

***In vitro* gene silencing**

H1299 cells were seeded in 96-well plates at a density of 8000 cells per well, 24 hours before transfection. Cells were treated with 20 µl of different polyplexes at a final siRNA concentration of 125 nM in FBS-containing medium. After four hours, medium was replaced, and cells were incubated for another 48 hours. Then, cells were washed and lysed in 100 µl reporter gene lysis buffer (Promega, Leiden, The Netherlands). After a freeze/thaw cycle, 50 µl lysate was mixed with 50 µl luciferase assay reagent (Promega) and luciferase activity was determined by measuring the luminescence for 10s at room temperature using a Fluostar OPTIMA microplate-based multi-detection reader equipped with a microinjector (Isogen Life Science). Luciferase activity of untreated cells was defined as 100% expression.

Statistical analysis

Results were analyzed using Student's t-tests to assess statistical significances. For multiple comparisons, ANOVA with a Bonferroni post-test was used.

RESULTS & DISCUSSION

Polymer synthesis

Two reducible poly(amido amine) copolymers were synthesized by Michael-type addition polymerization from *N,N'*-cystaminebisacrylamide (CBA), 4-amino-1-butanol (ABOL) and ethylene diamine (EDA) with or without α-amino-ω-hydroxy poly(ethylene glycol) (PEG-NH_2), to obtain a PEGylated and a non-PEGylated polymer (Figure 1). All polymers were obtained as a white to slightly yellow brittle material. The molar ratio between ABOL and EDA was found to be optimal at 75/25 for the unPEGylated polymer (9) and this ratio was used again here. The characteristics of the final polymers are listed in Table 1. The obtained ratio between ABOL, EDA and PEG, as well as the PEG content were determined from the NMR spectra. Molecular weights were determined by GPC. The obtained ratio between ABOL/EDA in the polymers deviated only slightly from the feed ratio and the obtained PEG fraction in the PEGylated polymer corresponded well with the aimed fraction. Based on the previous results in the p(CBA-ABOL/EDA) synthesis (9), all polymers are expected to be linear.

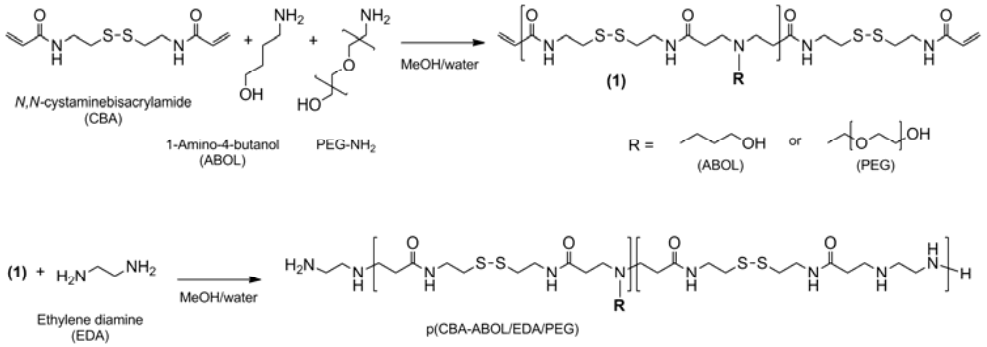

Figure 1. Schematic representation of the synthesis of the p(CBA-ABOL/EDA) and p(CBA-ABOL/EDA/PEG) copolymers.

Table 1: Synthesis characteristics

Polymer	Feed composition[a] ABOL/EDA/ PEG	Obtained composition[b] ABOL/EDA/ PEG	Wt % PEG[c]	M_w (kg/mol)	PDI	M_w PAA (kg/ mol)[d]
p(CBA-ABOL/EDA)	75/25/0	80/20/0	0 %	11.6	2.4	11.6
p(CBA-ABOL/EDA/PEG)	67/23/10	59/31/10	52 %	47.8	1.4	23.9

(a) Stoichiometrical feed ratio of monomers; (b) Determined by ^{1}H NMR; (c) PEG fraction of the polymer expressed as a weight percentage; (d) Molecular weight of the PAA fraction in the polymer, calculated from the wt % PEG.

Polyplex characteristics

In order to prepare polyplexes with various amounts of PEG in the formulations, mixtures containing different ratios of CBA-ABOL/EDA and CBA-ABOL/EDA/PEG were taken and mixed with siRNA. As it has previously been shown that PEG conjugation to polymeric vectors can interfere with polyplex formation and siRNA complexation, we first investigated the physicochemical properties of polyplexes with different PEG contents.

To determine the ability of the polymer mixtures to complexate siRNA, an agarose gel retardation assay was performed. Polyplexes were formed at polymer/siRNA (w/w) ratios of 0 (free siRNA), 3, 6, 12, 24 and 48. In the determination of the w/w ratios, the weight of PEG was not taken into account, thus the given ratios reflect the relative amount of cationic polymer in each formulation. For unPEGylated polyplexes, total siRNA retardation was observed at w/w ratios 12 and higher, indicating that the nucleic acid remained associated with the cationic polymers that do not migrate into the gel (Figure

2A, left). Complete retardation at this w/w ratio was also observed for polyplexes with 15, 30 and 45 wt% PEG (Figure 2B-D, left). For polyplexes that were prepared with 45 wt% PEG at the lower w/w ratios 3 and 6, siRNA was still visible in the wells, indicating that it was bound to the polymer but not completely shielded as ethidium bromide was still able to intercalate (Figure 2D, left).

The disulfide bridges in the poly(CBA-ABOL/EDA) polymers are expected to be cleaved in the cytosol because of its high redox potential as compared to the extracellular environment. To investigate whether the accessibility of these disulfide bonds for reducing agents like glutathione is restricted by PEGylation of the polyplexes, polyplexes were incubated for 1h at 37°C in the presence of 5 mM glutathione and subjected to electrophoresis. For all formulations, this incubation resulted in total release of siRNA from the polyplexes, indicating that the PEG chains do not interfere with polymer reduction (Figure 2A-D, right). Based on these results, further studies were performed with polyplexes prepared at polymer/siRNA w/w ratio 24.

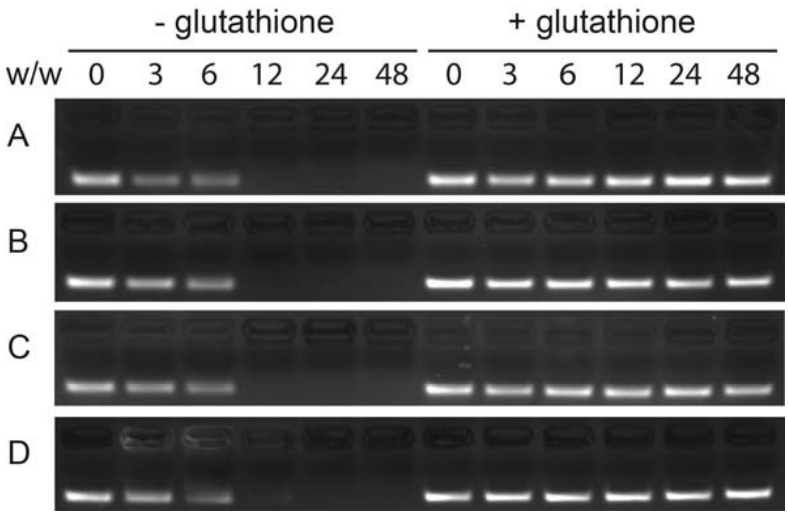

Figure 2. Agarose gel retardation of polyplexes prepared at w/w ratios 0 (siRNA only) to 48, after 1 h incubation at 37°C in the absence or presence of 5 mM glutathione. Polyplexes were prepared using polymers without PEG (A) or with a PEG content of 15 wt% (B), 30 wt% (C) and 45 wt% (D).

The influence of PEGylation on polyplex size and surface charge was investigated using dynamic light scattering and laser Doppler electrophoresis, respectively. UnPEGylated polyplexes prepared at w/w ratio 24 were around 160 nm in diameter and positively charged (± 20 mV) (Figure 3A,B). Polyplexes with 15 wt% or 30 wt% PEG were around the same size, while polyplexes formed with 45 wt% PEG were slightly smaller (Figure

3A). Smaller sizes for PEGylated polyplexes as compared to their unPEGylated counterparts has been shown before and was contributed to effective shielding and subsequent abolished aggregation (19, 20). Increased size for PEGylated polyplexes has also been reported, but always linked to perturbed nucleic acid condensation (17, 21). However, together with the data obtained from the agarose gel retardation assay, these results show that PEG conjugation of these polymers does not negatively affect polyplex formation. Furthermore, PEGylation substantially decreased polyplex surface charge down to ± 5 mV for polyplexes with 45 wt% PEG, which is in accordance with other reports on effects of polyplex PEGylation (17, 22, 23) (Figure 3B).

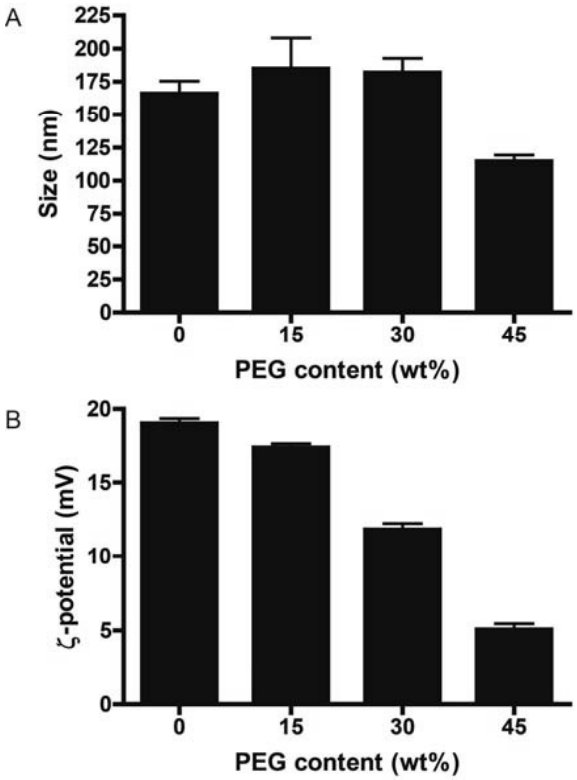

Figure 3. Size (A) and ζ-potential (B) of polyplexes prepared at polymer/siRNA (w/w) ratio 24 using polymers with increasing PEG content, as determined by dynamic light scattering and laser Doppler electrophoresis. Results are shown as mean ± SD for 3 individual measurements.

Polyplex stability

Systemic administration exposes polyplexes to physiological ionic strengths and anionically charged proteins, which can induce aggregation and destabilization by non-specific interactions (13). To investigate whether PEGylation prevents polyplex

aggregation at physiological salt concentrations, pre-formed polyplexes were incubated in PBS and size measurements were performed over time using dynamic light scattering. As expected, polyplexes without PEG showed a gradual increase in size to approximately 800 nm after 4 hours due to aggregation (Figure 4). Increasing the PEG content resulted in enhanced stabilization of the polyplexes. Polyplexes containing 15 wt% PEG displayed an aggregation profile similar to that of polyplexes without PEG, whereas a content of 30 wt% PEG substantially inhibited aggregation. 45 wt% PEG totally prevented aggregation up to 4 hours. Similar PEG-density dependent stabilization profiles were found for post-PEGylated cyclodextrin-based polyplexes formed with siRNA (24) or pDNA (25).

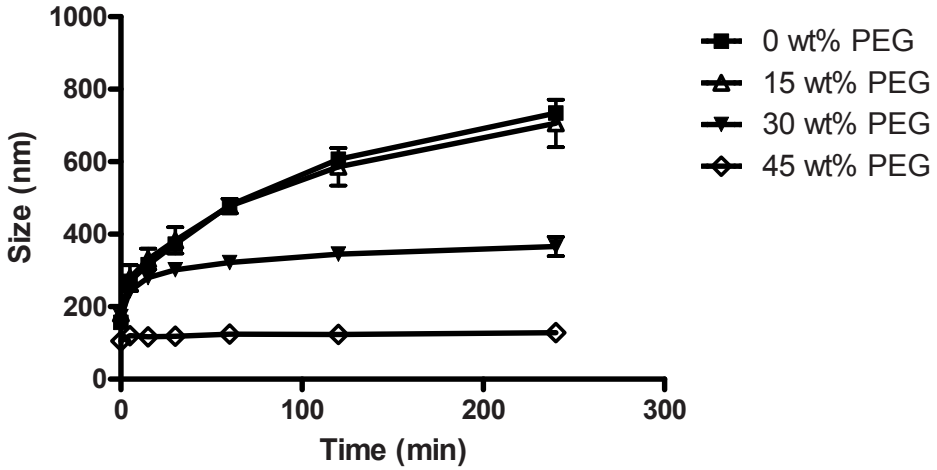

Figure 4. Polyplex stability in a physiological salt solution. Polyplexes were prepared at polymer/siRNA (w/w) ratio 24 using polymers with increasing PEG content. Particle sizes were determined by dynamic light scattering. Results are shown as mean ± SD for 2 individual measurements.

Next, we assessed effects of PEGylation on serum-induced aggregation by incubating pre-formed polyplexes in 90% FCS. Aggregation in terms of turbidity increase was significantly prevented by coupling of PEG to the polymeric structures and appeared to be more pronounced with increasing PEG content (Figure 5). Combined, these results indicate that PEGylation prevents polyplex aggregation in biological media.

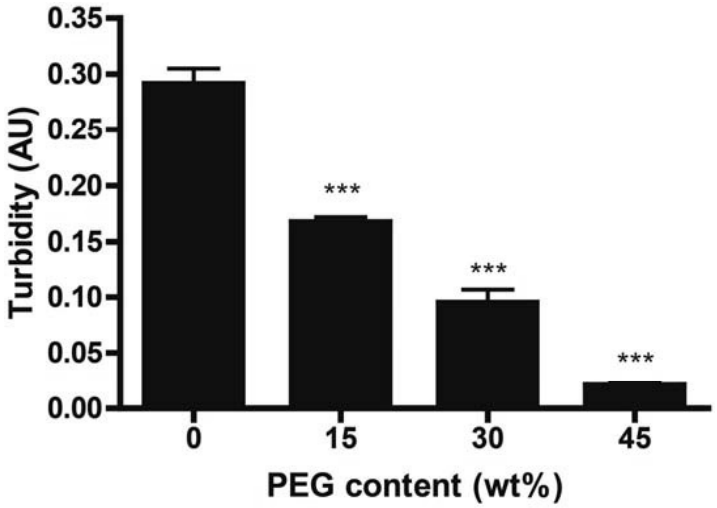

Figure 5. Serum-induced aggregation of polyplexes. Polyplexes were prepared at polymer/siRNA (w/w) ratio 24 using polymers with increasing PEG content. Aggregation in 90% FCS in terms of turbidity increase was quantified by absorbance detection at 595 nm. Results are shown as mean ± SD for n=3. Statistically significant differences versus polyplexes without PEG are denoted by *** ($p<0.001$).

Serum proteins as well as extracellular matrix components can however, besides inducing aggregation, also lead to vector disassembly (26). To evaluate possible effects of PEG on polyplex stability against competing anions, we tested the susceptibility of our polyplexes to heparin displacement. Heparin is one of the glycosaminoglycans (GAG), which are negatively charged polysaccharides that are the major components of the extracellular matrix in many tissues and are also found on the cell surface (27). Polyplexes with different PEG contents were prepared and subsequently incubated with increasing heparin concentrations. Polyplex dissociation in terms of siRNA release was shown by electrophoresis of the samples on an ethidium bromide-containing agarose gel (Figure 6). For unPEGylated polyplexes, dissociation occurred at heparin/siRNA (w/w) ratio 4 and higher. The presence of 15 wt% PEG did not alter the stability against heparin, but for polyplexes with higher PEG content a slightly decreased resistance against heparin competition was observed. Such a negative effect of PEG on polyplex stability was reported earlier for pEI-PEG/siRNA polyplexes and contributed to the weakened interaction with siRNA in the case of the PEGylated polymer as a result of decreased charge density (23).

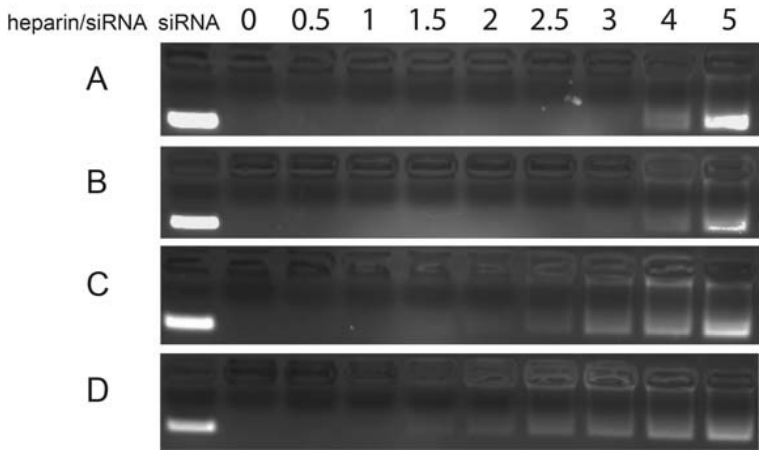

Figure 6. Heparin displacement assay. Polyplexes were prepared at w/w ratio 24 and incubated for 30 min at 37°C in the presence of increasing amounts of heparin, expressed as heparin/siRNA (w/w) ratio. Polyplexes were prepared using polymers without PEG (A) or with a PEG content of 15 wt% (B), 30 wt% (C) and 45 wt% (D).

Erythrocyte aggregation and hemolysis

Besides interactions with salt, proteins and/or the extracellular matrix, interactions with blood cells such as erythrocytes occur after systemic injection. Erythrocytes bear a negative surface charge and are known to interact with positively charged polyplexes, leading to aggregate formation and systemic toxicity (28). To evaluate the influence of PEG on polyplex-induced erythrocyte aggregation and hemolysis, polyplexes with different PEG content were incubated for 1h with freshly purified red blood cells. Erythrocytes were pelleted and visualized under a microscope, while the supernatant was used for measuring the degree of hemolysis as determined by absorbance detection at 550 nm. Control polyplexes were prepared using poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA), which were previously shown to induce severe erythrocyte aggregation (29). In our experiments, erythrocytes incubated with pDMAEMA polyplexes also displayed obvious aggregation as compared to cells incubated with buffer only. Polyplexes prepared from p(CBA-ABOL/EDA) polymers induced only mild aggregation, which was even further decreased using PEGylated polymers. For polyplexes containing 30 or 45 wt% PEG, no erythrocyte aggregation was observed (Figure 7A). A similar trend was observed for the degree of hemolysis. For polyplexes formed with pDMAEMA, approximately 16% hemolysis was observed, similar to what others have found for pEI polyplexes (30). A much lower hemolytic activity was measured for pCBA-ABOL/EDA polyplexes (approx. 4%). For PEGylated polyplexes, even less hemolysis was observed, down to less than 1% hemolysis for polyplexes with 45 wt%

PEG (Figure 7B). Together, these data indicate that PEGylated polymers have reduced interactions with blood components, which is in accordance with findings from others (13, 31).

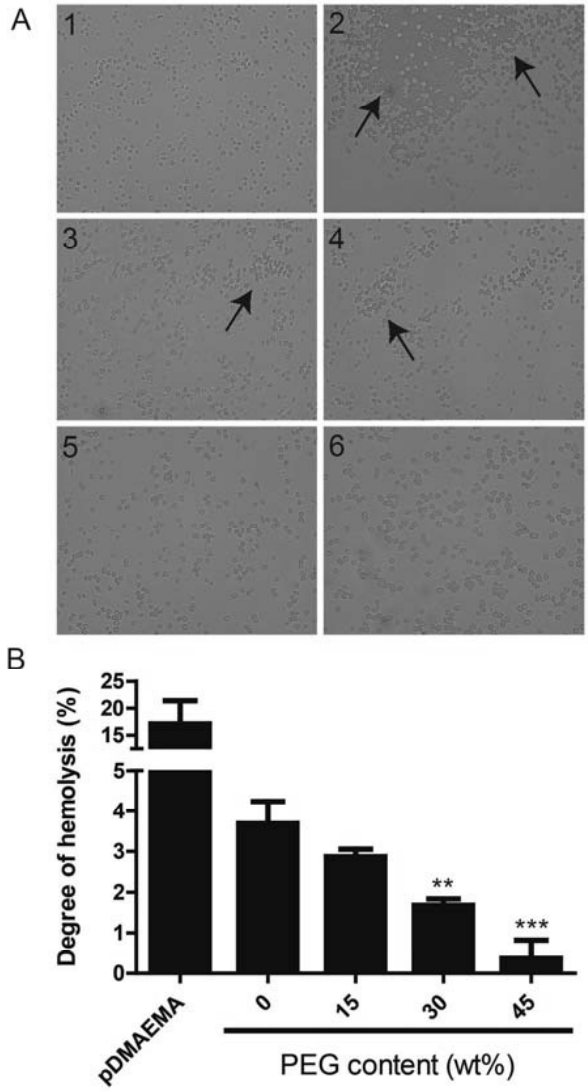

Figure 7. Polyplex-induced erythrocyte aggregation and hemolysis. Polyplexes were prepared using polymers with increasing PEG content and incubated with erythrocytes for 1 h at 37°C. pDMAEMA polyplexes were used as controls. (A) Microscopic image of erythrocyte aggregation. Aggregates are indicated with arrows. (1) HBG (2) pDMAEMA (3-6) p(CBA-ABOL/EDA) with increasing PEG content. (B) Degree of hemolysis as determined by absorbance detection at 550 nm. Results are shown as mean ± SD for n=3. Statistically significant differences versus polyplexes without PEG are denoted by ** ($p<0.01$) or *** ($p<0.001$).

Cellular uptake and gene silencing

To study the effects of PEGylation on intracellular uptake of polyplexes, H1299 cells were transfected with polyplexes with increasing PEG content, formulated using Alexa488-labeled siRNA. For quantification of siRNA internalization, fluorescence was determined in cell lysates 4 hr after transfection. 2% SDS was added to the lysis buffer to ensure complete polyplex dissociation, preventing possible quenching effects (18). As shown in Figure 8A, for all polymers, uptake was less than for control lipoplexes based on Lipofectamine 2000. Cellular uptake decreased with increasing PEG content in the polyplex. While the extent of uptake for polyplexes with 15 wt% PEG was similar to that of unPEGylated polyplex, uptake was significantly reduced for polyplexes containing 30 wt% PEG and almost completely inhibited for polyplexes containing 45 wt% PEG. Lower cellular uptake of polyplexes having increased PEG content was also confirmed by fluorescent microscopy (Figure 8B). As suggested by others, cellular uptake for PEGylated polyplexes is reduced because of PEG stealth effects, causing strongly reduced interaction with the cellular membrane (32, 33).

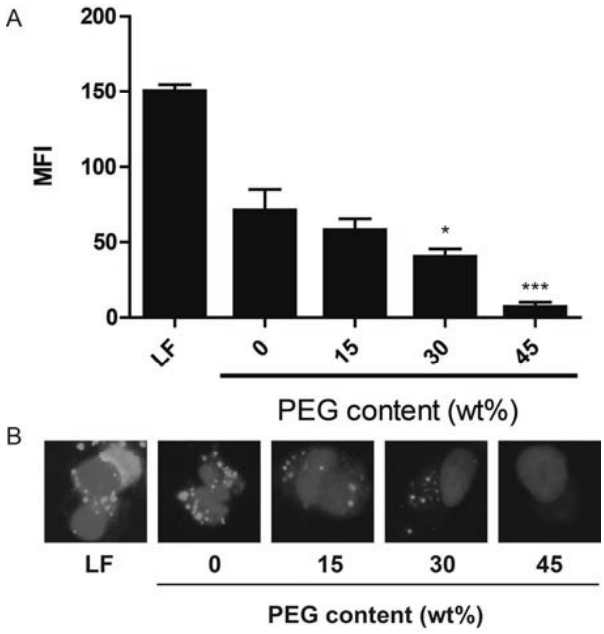

Figure 8. Cellular uptake of PEGylated polyplexes. (A) Mean fluorescence intensity (MFI) of H1299 cells determined 4 hours after transfection, corrected for protein content. Polyplexes were prepared using polymers with increasing PEG content and fluorescently labeled siRNA. Lipofectamine 2000 was used as control. Results are shown as mean ± SD for n=2-3. Statistically significant differences versus polyplexes without PEG are denoted by * ($p<0.05$) or *** ($p<0.001$). (B) Fluorescent microscopy pictures of H1299 treated cells. Nuclei are stained blue (DAPI), siRNA appears as green (Alexa-488).

Gene silencing potential of the PEGylated polyplexes was evaluated using H1299 cells that stably express the luciferase enzyme. As PEGylation reduced cellular uptake and hence is likely to reduce silencing efficiency, cells were transfected using a rather high siRNA concentration of 125 nM. At this concentration, transfection with Lipofectamine 2000 lipoplexes resulted in almost complete luciferase silencing. UnPEGylated polyplexes could efficiently inhibit luciferase expression, however this was combined with a relatively high cytotoxicity (approximately 50%), as indicated by the reduced luciferase expression of cells treated with control siRNA (Figure 9). The apparent toxicity of these polyplexes was not observed before (8, 9), and is likely caused by the increased polymer and siRNA dose which may lead in the presence of serum to particle aggregation and precipitation. As expected, with increasing PEG content, silencing efficiency was reduced, as well as cytotoxicity. In contrast, others have shown that PEGylation of pEI polyplexes leads to enhanced silencing activity, due to decreased polyplex stability and more efficient siRNA release (23). For our systems, siRNA release from the complex is most likely not a limiting factor for their biological activity, as a result of the triggered release mechanism due to the cleavage of the disulfide linkages in the polymer backbone. Interestingly, for polyplexes with high PEG content (30 or 45 wt%) cell viability was unaffected, while significant luciferase knockdown was still observed.

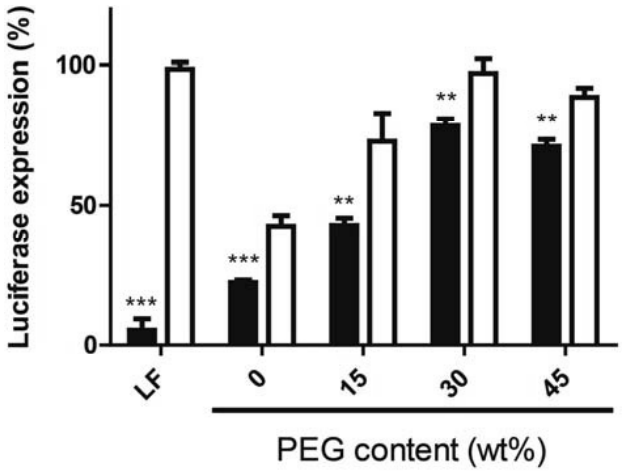

Figure 9. Gene silencing activity of PEGylated polyplexes. Luciferase expression of H1299 cells was determined 48 hours after transfection. Polyplexes were prepared using polymers with increasing PEG content and luciferase siRNA (black bars) or non-silencing control siRNA (white bars). Lipofectamine 2000 was used as control. Results are shown as mean ± SD for n=3. Statistically significant differences of luciferase siRNA versus non-silencing control siRNA are denoted by ** ($p<0.01$) or *** ($p<0.001$).

Although the knockdown efficacy of PEGylated polyplexes *in vitro* is low as compared to Lipofectamine 2000 or unPEGylated polymers, improved *in vivo* delivery due to decreased aggregation and increased circulation time can be expected. Addition of targeting ligands to the distal ends of the PEG chains can be expected to boost their cellular uptake, resulting in increased silencing efficiency, and this is considered as the next step in the development of targeted delivery systems for siRNA (34).

CONCLUSIONS

In this study, the synthesis of a novel PEGylated polymer based on biodegradable poly(amido amine)s with disulfide linkages in the backbone was described for the delivery of siRNA. Polyplexes with various PEG contents were prepared by mixing the PEGylated polymer with its unPEGylated counterpart prior to polyplex formation. PEGylation decreased polyplex surface charge, increased their stability against salt and serum and decreased polyplex interactions with erythrocytes. Controversially, PEGylated polyplexes showed decreased resistance against heparin displacement. Cellular uptake was lower for polyplexes with increasing PEG content, which resulted in reduced gene silencing efficiency, but also reduced toxicity. Polyplexes with PEG contents of 30 and 45 wt% showed significant silencing efficiency in the absence of toxicity, which makes them promising carriers for delivery of siRNA *in vivo*. The addition of targeting ligands on the PEG chain ends is expected to further improve their cellular uptake and silencing potential.

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Chapter 7

Lipid-based formulations for siRNA delivery

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INTRODUCTION

RNA interference (RNAi) is an evolutionary conserved process for post-transcriptional silencing in many species, including mammalians (1, 2). The physiological functions of RNAi are viral defense, transposon silencing and regulation of developmental pathways. RNAi is mediated by small interfering RNA (siRNA) molecules (3), which are able to interact with several proteins to form a multienzyme complex including Argonaute 2 (AGO2) and the RNA-Induced Silencing Complex (RISC) (4-6). Upon unwinding of the siRNA, the antisense strand guides the AGO2-RISC complex to complementary mRNA, which is subsequently cleaved (3, 7).

Using this machinery, introduction of synthetic siRNA molecules of 21-23 nucleotides into cells has been shown to result in sequence-specific silencing of genes at low concentrations compared to other antisense drugs (2). In addition to its use in functional genomics, siRNA hold great promise as a therapeutic agent with wide applications for treatment of severe and chronic diseases (8-11). The huge potential of RNAi for human therapy is demonstrated by the fact that several siRNA-based drugs are already in clinical trials (some in phase III). However, despite these high expectations, a number of delivery hurdles has to be overcome before the therapeutic potential of siRNA can be realized. Firstly, naked siRNA merely has a half-life of several minutes in vivo due to degradation by nucleases and excretion in the kidneys, which is an insufficient length of time to induce silencing (12). Furthermore, siRNA needs to reach the specific cells that are actively translating the targeted mRNA. Finally, cell membrane passage of a relatively large and highly charged polyanion like siRNA represents a major obstacle. The major challenge of bringing RNAi from laboratory tool to a therapeutic strategy is therefore to develop effective delivery systems that can increase stability, cellular targeting and cell membrane passage of siRNA, and thereby reduce the amount and costs of siRNA required for therapeutic efficacy.

To date, several delivery systems for siRNA have been described, including viral vectors and non-viral vectors. For therapeutic use, non-viral vectors are preferred by virtue of their ease of synthesis, low immune response, and benefits in terms of safety. Among the wide variety of non-viral vectors, lipid-based carriers have been the most extensively described in both *in vitro* and in vivo studies.

The majorities of lipids applied for siRNA delivery are cationic and interact readily with negatively charged siRNA molecules to form so-called lipoplexes, which are heterogeneous and metastable aggregates. However, also neutral and even negatively charged lipids have been used. Delivery systems based on these lipids rely on entrapment of siRNA in the aqueous compartment of liposomes to form lipid-based nanoparticles (13). All these systems are able to protect siRNA from enzymatic degradation (12, 14).

Moreover, the hydrophobization of the siRNA by the lipids promotes passage across cellular membranes.
Despite these attractive features of lipid carriers for nucleic acid delivery in general, no impressive results have been obtained in clinical trials to date due to limited delivery efficiency in vivo. Additionally, the occurrence of non-specific immune-stimulating effects of lipids as well as siRNAs has retarded the development of more efficient carrier systems.
In this chapter an overview is provided of currently employed lipid-based delivery systems for siRNA, and of recent research into improvements of cellular delivery by enhancing target cell uptake and redirecting intracellular trafficking routes. In addition, the role of lipids in immune activation is discussed, and possible ways to overcome the response are highlighted.

CATIONIC LIPID-BASED DELIVERY SYSTEMS FOR NUCLEIC ACID-BASED DRUGS

The multivalency of cationic liposomes provides for electrostatic interactions with the negatively charged phosphate backbone of nucleic acids, resulting in condensation of the nucleic acid into particles, also called lipoplexes (15-17). Preformed cationic liposomes are among the most efficient *in vitro* transfection reagents for nucleic acids/siRNA. Their high efficiency is partly caused by the large amounts of siRNA that can be complexed by cationic lipids, compared to passive loading in charge neutral liposomes (12, 14). Besides condensation of nucleic acids, cationic lipids serve to protect nucleic acids against degradation by nucleases and provide the complexes with a net positive charge, enabling efficient binding of the complexes to negatively charged heparan sulfate proteoglycans present on cell surfaces (18-20). Transfection-competent lipoplexes possess an ordered structure with irregular morphology, which for cationic liposome-DNA complexes consists of DNA coated by cationic lipid monolayers and arranged on a so-called two-dimensional hexagonal lattice (21). Lamellar liposome-DNA complexes are less efficient for transfection. Surprisingly, a study with siRNA showed that lamellar cationic lipid-siRNA complexes were superior for gene silencing compared to inverted hexagonal complexes, despite the general similarity between the structure of lipid-DNA and lipid-siRNA complexes (22). This indicates that important aspects of lipoplex-mediated siRNA delivery differ significantly from lipoplex-mediated DNA delivery.
To date a variety of cationic lipids have been synthesized for *in vitro* transfection purposes but most, if not all, of them have failed in clinical trials due to poor in vivo performance and serious drawbacks, such as low in vivo transfection efficiency, toxicity problems associated with immune activation and unfavorable biodistribution. Lipoplexes are rapidly cleared upon systemic administration due to accumulation in the capillary bed

of first-pass organs such as the lungs (23, 24). First-pass clearance is followed by hepatic elimination of the majority of the lipoplexes 60 minutes after injection due to redistribution from the lungs to the phagocytic Kupffer cells of the liver (25). Strong interactions between the cationic surface of lipoplexes and plasma proteins prime the lipoplexes for this rapid elimination by the reticuloendothelial system (RES) (26, 27).
To circumvent the shortcomings of cationic lipids, titratable, ionizable lipids have been developed (28, 29). The lipids are strongly charged at reduced pH, offering the possibility of efficient complex formation, but are more neutral at physiological pH. This approach still benefits from the charge interaction at reduced pH but avoids the unfavorable biodistribution of charged systems by changing the pharmacokinetics, enabling passive targeting of neutral, appropriately sized (100 nm) liposomes to pathological tissue via the enhanced permeability and retention effect (EPR) upon systemic administration (30). Another option is to remove residual cationic surface charge upon complex formation (31) or to shield the charge of lipoplexes by covering them with an additional, neutral membrane bilayer, so-called wrapped liposomes (32, 33).

HELPER LIPIDS

Some cationic lipids possess intrinsic fusogenic properties, enhancing the membrane fusion and intracellular delivery of lipoplexes (29, 34, 35). Above the phase transition temperature, some lipids can adopt a reverse hexagonal H_{II} phase that is highly fusogenic (21, 29, 36). For example, for titratable cationic lipids it has been shown that the fusogenicity of the liposomal system increases as the lipid becomes less saturated (29). This suggests that a more flexible bilayer structure is important for the propensity to destabilize cell membranes. However, in addition to cationic lipids, so-called helper-lipids are often included in liposomal formulations for efficient delivery (34, 35, 37, 38). Non-bilayer forming lipids like dioleyl phosphatidylethanolamin (DOPE) can form a reverse hexagonal H_{II} phase that promotes destabilization of the lipid bilayer and fusion with the target cell membrane (21). In vivo, the increased transfection competency of fusogenic systems might, though, be counterbalanced by their increased propensity to interact with components in the circulation, resulting in stability problems. However, for siRNA, a recent study indicates that inclusion of DOPE in cationic liposomes does not increase the transfection efficiency but rather causes increased toxicity and lower target-specific gene silencing (22), indicating that DOPE is not beneficial for siRNA delivery but only for plasmid delivery.

LIPOPOLYMERS

Another way to circumvent the fast clearance of lipoplexes and delay the uptake of lipid-based nanoparticles into the RES is to shield and stabilize the cationic lipid bilayer with polymers like poly(ethylene glycol) (PEG) (39-41), which allows for passive targeting to vasculature at sites where the endothelium is leaky (EPR effect) due to increased circulation time upon systemic administration. However, PEG prevents the cell association, uptake, and membrane destabilization necessary for endosomal escape, resulting in low transfection efficiency (41-43). This possesses a contradictory dilemma of particle stabilization and charge shielding versus target cell uptake, drug release, and membrane destabilization.

Therefore, coatings that may be shed have been developed; these are dissociated from the carrier upon arrival at the target site, converting the delivery system from a stable particle to a transfection-competent complex, thereby facilitating drug release and/or target cell interaction processes (44). One such coating is diffusible PEG-lipids that can dissociate from the carrier, with dissociation constants depending on the length of the lipid anchor (45). Diffusible PEGs have been incorporated into siRNA-containing liposomal systems (Table 1) called stabilized siRNA lipid particles (SNALPs) (12, 14, 46). Zimmermann et al. observed dose-dependent silencing of apolipoprotein B (ApoB) in the liver of cynomolgus monkeys after a single intravenous (i.v.) injection of SNALPs encapsulating siRNA directed towards ApoB (14). The SNALP liposomes contained distearoyl phosphatidylcholine (DSPC), cholesterol, the cationic and fusogenic lipid DLinDMA, and the diffusible lipopolymer PEG-C-DMA, prepared by a carefully controlled stepwise ethanol dilution method (47). The plasma half-life of SNALP-encapsulated siRNA was 38 minutes in mice (14), compared to less than 2 minutes for naked siRNA (12). The liposomal siRNA showed 50% silencing of apoB messenger at 0.1 mg/kg SNALP-formulated ApoB-siRNA, whereas a 100 mg/kg dose was required to obtain comparable levels of efficacy with a cholesterol-conjugated ApoB siRNA, which corresponds to a 1000-fold increase in potency (48). No immune stimulation or toxic effects were observed.

An alternative to diffusible PEGs involves the use of stimuli-responsive lipid-polymer conjugates with a chemically labile linker between the lipid anchor and the polymer. An example is incorporation of an acid labile PEG-diorthoester lipid conjugate into the bilayer that is destabilized at the low pH found in the endosomes, facilitating the release of entrapped drug (49, 50).

Table 1: Examples of liposomal siRNA delivery systems

Lipid composition	Target gene	Formulation Method	Size (nm)	Encapsulation (%)	Ref.
DSPC:Chol:PEG-C-DMA:various cationic lipids	Luciferase (*in vitro*)	Ethanol dilution - SNALP	132-182	67-85	(29)
DSPC:Chol:PEG-C-DMA:DLinDMA or DODMA	B-galactosidase (*in vitro*)	Ethanol dilution - SNALP	100-130	90-95	(73)
DSPC:Chol:PEG-C-DMA:DLinDMA	Hepatitis B virus (in vivo)	Ethanol dilution – SNALP	140 ± 12	93 ± 3	(12)
DSPC:Chol:PEG-C-DMA:DLinDMA	Apolipoprotein B (in vivo)	Ethanol dilution – SNALP	73-83	92-97	(14)
DSPC:Chol:PEG-C-DMA:DLinDMA	Polymerase (L) gene of Ebola virus (in vivo)	Ethanol dilution – SNALP	71-84	90-95	(46)
DSPC:Chol:PEG-C-DMA:DLinDMA	Apolipoprotein B (in vivo)	Ethanol dilution - SNALP	100-130	90-95	(75)
DOTAP:DOPE:TfRscFV	None	Ethanol injection	211 ± 18	ND	(51)
DOTAP:DOPE:TfRscFV	HER-2 (in vivo)	Ethanol injection	100	ND	(52)
Gal-C4-Chol/DOPE	Ubc13 gene (in vivo)	Passive encapsulation	50 ± 4	ND	(54)
(Diethylaminoethyl) Carbamoyldioleoyl glycerol:PC: Lactosylated PE	Hepatitis C virus (in vivo)	Passive encapsulation	ND	ND	(53)
DOTAP:Chol:DNA: protamine:DSPE-PEG-anisaminde	None	Passive encapsulation	120-150	>95	(58)
DOTAP:Chol:DNA: protamine:DSPE-PEG-anisamide	Epidermal growth factor receptor (in vivo)	Passive encapsulation	120-150	>95	(57)
EPC:Chol:PEG-PE:DOTAP	Human double minute gene 2 (HDM2) (*in vitro*)	Passive encapsulation	50-200	ND	(61)
PC:DMPA:Chol	None	Passive encapsulation	880	ND	(85)
DOPC:Tween 20	EphA2 (in vivo)	Passive encapsulation	ND	65	(63)
DOPC:Tween 20	Focal adhesion kinase (in vivo)	Passive encapsulation	ND	ND	(64)
DPPG:DPPC:DPPE-PEG	Enhanced green fluorescent protein (*in vitro*)	Reverse-phase evaporation	60-90	7-14	(62)
DOTAP:DOPE: Transferrin	c-Jun/luciferase (*in vitro*)	Passive encapsulation	328-926	90	(86)

TfRscFV: anti-transferrin receptor single-chain antibody fragment

ACTIVE TARGETING

Active targeting strategies have been applied to provide sterically stabilized, long-circulating systems with increased affinity for target cells. The cellular binding and uptake of liposomes can be increased by attaching ligands for internalizing receptors tothe distal ends of PEG-chains. Examples of commonly used targeting moieties are peptide and protein ligands like antibodies and peptides (51, 52), vitamins like folate and glycolipids (53, 54). Active targeting serves to increase the therapeutic index of drugs by increasing the fraction of drug reaching the target site, and by reducing the fraction of drug reaching non-target sites and toxicological targets. Disadvantages of active targeting approaches are though increased costs, manufacturing complexity, and increased risk of immunogenicity. Development of a post-insertion technique has allowed for insertion of ligand-PEG-lipid conjugates into preformed liposomes (55).

A few studies have been published where siRNAs have been successfully delivered in vivo with actively targeted liposomal formulations. Pirollo et al. formulated siRNA into immunoliposomes targeted to upregulated transferrin receptors present on tumor cells via an anti-transferrin receptor single-chain antibody fragment (51, 52, 56). A histidine-lysine peptide was included in the complex to enhance endosomal release. High levels of siRNA were delivered specifically to tumors upon systemic administration (51), and were able to silence the target gene HER2 and specifically inhibit tumor growth (52).

Huang and co-workers developed self-assembling siRNA nanoparticles prepared by mixing carrier DNA, siRNA, protamine, and lipids (DOTAP and cholesterol), followed by post-modification with the ligand anisamide coupled to PEG-lipid (57-59). These particles are also called LPD nanoparticles. Anisamide is a ligand for the sigma receptor present on certain tumor cells. As much as 70-80% of the injected siRNA per gram of tissue accumulated in the tumor in a mouse xenograft model, and 40% tumor growth inhibition could be achieved.

Other ligands include cell-penetrating peptides (CPPs) (60) that can increase the transfection efficiency upon association to siRNA-liposome complexes (61).

NEUTRAL AND ANIONIC LIPID-BASED DRUG DELIVERY SYSTEMS

Liposomes composed of neutral or zwitterionic lipids have much longer circulation times, lower toxicities, and very different clearance profiles compared to cationic particles. Neutral liposomes have been successfully applied as carriers for anti-cancer drugs and antibiotics, to achieve lower toxicities, altered distributions, and higher drug efficacies than unencapsulated drugs (30). Neutral and anionic liposomes have, however, the disadvantage compared to lipoplexes that siRNA cannot be entrapped efficiently via

electrostatic interactions, and their transfection efficiencies are much reduced compared to lipoplexes (62).
Landen et al. used a liposomal formulation consisting of DOPC and Tween 20 (Table 1) to encapsulate an siRNA targeting the oncoprotein EphA2 (63). The formulation was able to silence EphA2 gene expression 48 hours after administration and could inhibit tumor growth in an orthotopic mouse model of ovarian cancer. The same formulation was successfully applied to silence the focal adhesion kinase expressed by ovarian cancer cells, resulting in significantly reduced tumor growth in mice (64).
Another option is to pre-condensate siRNA with a polycation, followed by coating with negatively charged or neutral lipids (32, 33, 58, 65). The polycation could be a cationic liposome or a polymer like poly(ethylene imine), protamine sulfate or polylysine.

MECHANISMS OF INTERNALIZATION

Most studies regarding internalization routes of lipid-nucleic acid complexes have been performed with plasmid DNA. Although it seems likely that siRNA-lipoplexes essentially follow the same fate as their plasmid DNA counterparts, formal studies in this field are still lacking.
For cationic lipid-delivered siRNA five internalization routes seem most probable: (i) Clathrin-mediated endocytosis, (ii) caveolae-mediated endocytosis, (iii) macropinocytosis, (iv) phagocytosis and (v) receptor-mediated endocytosis. Although fusion of lipoplexes at the cell surface could, in principle, occur, the majority of studies indicate that a certain kind of endocytosis contributes most to intracellular delivery. Size as well as composition of the siRNA-lipid complex can influence the route of internalization.
Large lipoplexes (in the range of 0.2 μm to 0.5 μm) seem to be preferentially taken up through receptor- and clathrin-independent endocytosis. The exact nature of this uptake route is debated. Matsui et al. suggested phagocytosis of large lipoplexes occurred in lung epithelial cells (not considered to be specialized phagocytes), where the uptake could be inhibited by cytochalasin B and potassium depletion (66). Rejman et al. suggested that larger particles are primarily taken up by caveolae (67). This would allow for targeting of particles through a non-degradative pathway, as caveolae-mediated endocytosis is a pathway that is not coupled to acidification of the compartment and concomitant lysosomal fusion.
In contrast, smaller complexes (< 0.2 μm) are primarily internalized via coated pits through a non-specific, clathrin-dependent process (68). This route of endocytosis involves an intracellular pathway, in which lysosomes ultimately fuse with internalized vesicles, resulting in degradation of their contents.

For the remaining two internalization routes, modification of the lipoplex surface seems required. Octa-arginine modified lipoplexes appeared to be taken up by macropinocytosis (69). Receptor-mediated endocytosis can be enforced by coupling the matching targeting ligand to the lipoplex surface. This usually requires shielding of the net cationic charge of the complex by polymers such as PEG to optimally benefit from the receptor-ligand interaction. Interestingly, cholesterol-conjugated stabilized siRNA present on the outside of low density lipoproteins was internalized via the siRNA-specific SID-1 receptor in HepG2 cells, which led to functional silencing (70). This indicates that, for certain cell types, delivery to the cellular membrane may be sufficient, after which dissociation of siRNA and lipid may occur followed, by translocation of siRNA over the cytolemma.
After endocytic uptake, a membrane barrier still remains that separates the siRNA from the cytoplasm, where the RNAi-machinery is located. Caveolar uptake provides limited environmental triggers for crossing the caveolar membrane but constitutes a non-degrading environment. In contrast, for other routes acidifying conditions in the late endosome can provide a cue for fusion of the lipoplex with the endosomal membrane. This may be achieved by inclusion of peptides or lipids that change conformation upon protonation.

IMMUNE ACTIVATION

Although viral vectors have been shown to have superior abilities to transfect cells, they are often not the best choice for siRNA delivery for therapeutic purposes. The presence of specific immune reactions against viral particles has forced researchers to look for alternative agents for delivery. For a long time, non-viral vectors were considered to be inert, thereby avoiding these inflammatory responses. Although they have now become the first option as delivery systems, more recent studies on immune activation of lipidic particles indicate that this might not be the case at all.
For instance, Mui et al. found that immune stimulation by a CpG-containing oligodeoxynucleotide (ODN) is enhanced when delivered in lipid particles (71). The therapeutic benefit from ODN-containing immune stimulatory sequences like CpG in animal models of cancer and infection had already been demonstrated. Mui et al. showed that after administration of CpG ODN, encapsulated in lipid particles, plasma concentrations of cytokines like interleukin (IL)-12, interferon (IFN)-γ, IL-6, monocyte chemoattractant protein-1 and tumor necrosis fator (TNF)-α in mice were greatly increased compared with free CpG ODN. They concluded that the increased immunogenicity was largely a result of enhanced delivery of CpG ODN to macrophages and other antigen-presenting cells by the lipid particles. Indeed, liposomes are removed from the circulation by macrophages and monocytes. An increased immunostimulatory activity of CpG ODN as a result of formulation in liposomal nanoparticles was also

found by De Jong et al (72). They investigated the immunopotency and also anti-tumor activity of subcutaneously administered CpG ODN in animal models of cancer.
A lipid-mediated immune reaction has also been shown for delivery of siRNA. Although not generally accepted, it seems that transfection of siRNAs results in activation of the interferon system (73). Interferons are cytokines that are part of the innate immune response that can be triggered by double stranded RNA molecules through the binding to Toll-like receptors (TLR). Ma et al. investigated this response in mice and found that although intravenous administration of a specific siRNA in this case was essentially inert, injection of cationic lipid-siRNA complexes induced a strong interferon response (74). It was also suggested that the mechanism behind this increased reaction is enhanced uptake of siRNA by immune cells.
In addition to the immune activation induced by the lipid vector, certain siRNA sequences have also been shown to induce activation of the innate immune system, triggered primarily by the recognition of dsRNA by TLR7 (73, 75-77). Some TLRs, including TLR7, are localized in endosomes, and immune responses elicited by siRNA are greatly potentiated by delivery systems like liposomes that facilitate intracellular delivery to endosomes. It seems possible, though, to design non-inflammatory siRNAs by avoiding GU-sequence motifs and by incorporation of 2´-O-methyl uridine or guanosine nucleosides into one strand of the siRNA duplex (75).
Lipid carriers are not only able to increase immune reactions caused by other agents, as mentioned above, but can also be immunogenic by themselves. Therefore, liposomes are used as immunological adjuvants in vaccine formulations (78-80). The first indications for undesired immune reactions of lipid-based carriers for nucleic acid delivery arose from microarray studies that examined the effects on gene expression profiles of target cells. In one study, Omidi et al. investigated the effects of two lipid-based carrier systems, Lipofectin and Oligofectamine, on gene expression profiles of human A431 epithelial cells (81). They hypothesized that incubation of cells with cationic lipid formulations had an effect on their global gene expression, based on a previous study performed by Petch al. (82), showing that changes in non-target gene expression following treatment of cells with oligonucleotide-lipid complexes was not the result of the oligonucleotides alone. Using cDNA microarray expression profiling, they showed that treatment with Lipofectin or Oligofectamine resulted in a more than twofold altered expression of 6% or 17% of the investigated genes, respectively. These changes led to downstream functional consequences such as increased apoptosis (81).
A possible explanation for the demonstrated immunostimulation of antigen-presenting cells by cationic lipids alone was published by Vangasseri et al. (83). In their study on anti-tumor immune responses through delivery of a human papillomavirus-E7 epitope antigen to dendritic cells, they found that not only the plasmid DNA but also the cationic liposomes that were used to deliver the antigen led to expression of immunostimulatory

molecules (83). Interestingly, stimulation of dendritic cells occurred via a different mechanism in both situations. Furthermore, the ability of lipids to activate an immune response appeared to be structure-specific. Both head group and hydrocarbon chain structure were found to have an influence on the immunological outcome. According to the authors, these findings point towards the possible existence of a cationic lipid binding protein, which can activate cellular signaling cascade(s) resulting in the production of (co-) stimulatory molecules (83).

Despite the fact that the pathway by which lipids stimulate the immune system is still largely unknown, the first studies on how to decrease the inflammatory response to these vectors have already been published. Liu et al. tried to decrease the inflammatory response to DOTAP liposomes containing plasmid DNA by incorporating two small molecules, known for their ability to specifically inhibit the NF-κB or MAPK inflammatory pathway, into the formulation (84). These small molecules, including chenodeoxycholic acid, capsaicin and the MAP kinase inhibitor PD98059, were efficiently encapsulated into the lipoplexes and were able to significantly decrease the TNF-α response to the liposome formulation. The gene expression levels were found to be similar for all kinds of lipoplexes. The results also suggest the possibility to reduce the immune response by an even greater extent by incorporation of several small molecules that inhibit different immune activation pathways (84).

Although the long-term effects of lipid-induced gene expression are still unknown,the results from an increasing number of studies suggest that lipid-based vectors do induce an, often undesired, rapid immune reaction. This finding might have a great influence on the development of non-viral vectors, because their inertness towards antigen-presenting cells has long been their greatest advantage in comparison to viral delivery systems.

CONCLUSIONS

Multiple in vivo experiments have consolidated the promising therapeutic efficacy of siRNA, but there is an urgent need for the development of novel carrier systems for siRNA. Liposomes are versatile delivery systems that can be designed and provided with properties needed for successful delivery of siRNA for a variety of pathological conditions. These properties include siRNA protection, decreased elimination, specific cellular targeting, increased membrane passage and siRNA release. Such systems serve to improve the therapeutic outcome by reducing the dose and, thereby, the costs of an expensive drug like siRNA. Multicomponent and/or multilayered systems seem to be required to equip the delivery system with the often contradictory requirements for in vivo administration, although simple formulations are highly desired for clinical applications. The complex composition of many lipid-based carrier systems demands a

careful characterization of the formulations for a predictive and reproducible therapeutic outcome.

In addition, the development of novel, optimal and non-cationic lipid-based carrier systems that are safe and well tolerated, possess favorable pharmacokinetics, intracellular/cytoplasmic delivery and are stable upon manufacture is highly requested. To optimally tailor the lipidic delivery systems for siRNA better insight is needed into the mechanism of cell delivery. More specifically, pharmacokinetics, the nature of cell surface interaction of siRNA-lipid complexes, routes of internalization, passage of intracellular membranes and mechanisms of immune activation need further clarification. For siRNA in particular, increased knowledge on the off-target effects of the delivery systems is of special interest, since siRNA is currently being investigated in clinical trials. More research is required to elucidate the mechanism(s) behind lipid-based immune reactions and how to avoid them. Increased knowledge will help the design of better and safe lipid-based delivery systems for siRNA in the future.

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Chapter 8

Targeted delivery of small interfering RNA to angiogenic endothelial cells with liposome-polycation-DNA particles

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ABSTRACT

Angiogenesis is an attractive target for cancer therapy, due to its central position in tumor growth and development. Vascular Endothelial Growth Factor (VEGF) and its receptors (VEGFRs) play a key role in the angiogenic process. A promising strategy for targeting VEGF-mediated angiogenesis is RNA interference (RNAi) using short interfering RNA (siRNA). However, for efficacious RNAi a well-designed siRNA delivery system is crucial. Liposome-Polycation-DNA (LPD) particles form a promising system for siRNA delivery to tumors. In order to target angiogenic endothelial cells, LPD particles may be modified with a targeting ligand, such as a cyclic Arg-Gly-Asp (RGD) peptide that specifically binds to integrins expressed on tumor-associated endothelial cells. In the current study, RGD-targeted PEGylated LPD particles containing VEGFR-2 siRNA were prepared and optimized with respect to their size and charge by varying protamine content, carrier DNA content for stronger complexation, and PEGylation density. The size of the optimized particles was around 200 nm and the ζ-potential was approximately +20 mV. The uptake and silencing efficacy of the RGD-targeted PEGylated LPD particles were evaluated in H5V cells (murine endothelial cells) and Human Umbilical Vein Endothelial cells (HUVECs). When compared to non-targeted LPD particles, enhanced uptake and silencing of VEGFR-2 expression was observed for RGD-targeted PEGylated LPD particles. In conclusion, the RGD-targeted PEGylated LPD particles containing VEGFR-2 siRNA presented here may be a promising approach for targeting VEGF-mediated angiogenesis in cancer therapy.

INTRODUCTION

Since 40 years, angiogenesis, the process of formation of new blood vasculature, has been recognized to be a prominent factor in the development of solid tumors (1). Initiated by the discovery of tumor angiogenic factor (TAF) by Folkman et al., a serum protein currently known as vascular endothelial growth factor (VEGF), the complex process of neovascularization has since then been regarded as an attractive target for cancer therapy (2, 3).

Driven by a shortage of oxygen, the angiogenic sprouting of microvessels is initiated and coordinated by factors excreted by various cell types, such as tumor cells, vascular endothelial cells and platelets (4, 5). VEGF, which is upregulated in cells exposed to hypoxic conditions such as in the tumor environment, and the VEGF receptor family play a key role in the process of angiogenesis (6-8). Of the three VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3) that have been described, VEGFR-2 (also referred to as fetal liver kinase-1 (Flk-1) in mice, and kinase insert domain receptor (KDR) in humans), which is expressed by vascular endothelial cells and endothelial progenitor cells, is regarded as the most important regulator of tumor neovascularization (7, 9). Upon binding of VEGF, VEGFR-2 undergoes dimerization and autophosphorylation, initiating several intracellular signaling cascades, such as the Rho GTPase pathway, which eventually translate to cellular activation of angiogenic processes (6, 10, 11). Because of their dominant role in angiogenesis and therefore tumor development, VEGF and VEGFR-2 have become attractive targets in cancer therapy. Currently, several inhibitors of VEGF-mediated angiogenesis are clinically approved or in development in the USA and Europe. For example, bevacizumab, a humanized monoclonal antibody against VEGF, and VEGF-TRAP, a soluble fusion protein of VEGFR-1, VEGFR-2 and the Fc segment of human IgG1, bind VEGF with high affinity, thereby preventing the binding of VEGF to VEGFR-2 and thus inhibiting the onset of angiogenesis and the establishment and progression of tumors (12, 13). From another perspective, kinase inhibitors like sunitinib may target the VEGFR-pathway in endothelial cells by inhibiting signaling through several tyrosine kinases, such as the VEGFR-2 (14). Compared to other tumor-associated cells, the angiogenic endothelial cells form a more straightforward target for inhibition of angiogenesis. They are readily accessible for intravenously injected therapies and they possess a higher genetic stability, which reduces the risk of drug resistance (4). However, despite initial promising results, current anti-angiogenic drugs offer modest clinical therapeutic efficacy and display serious side effects (15). An alternative strategy to interfere with VEGFR-signaling in endothelial cells is silencing of VEGFR-2 expression by RNA interference (RNAi) (16). Posttranslational gene silencing RNAi has become a rapidly growing field of research since its discovery in *Caenorhabditis elegans* by Andrew Fire and Craig Mello in 1998

(17, 18). Although it is regarded as a conserved endogenous cellular mechanism that protects against viruses, RNAi can be exploited therapeutically, by introducing short interfering RNA (siRNA) in the cellular cytoplasm to suppress the expression of specific genes, such as those involved in angiogenesis (19, 20). Since siRNAs are large, negatively charged molecules, preventing them from passing the cellular membrane, and rapid degradation and clearance upon systemic injection is inevitable, delivery systems that protect siRNAs and deliver them to the site of action are required (21). Over the years, many types of delivery systems have been developed, including liposomes, lipoplexes, peptide complexes and polymers (22-25). Although up till now some successes have been booked with RNAi, toxicity and poor stability of the delivery systems have remained hurdles towards bringing siRNAs into the clinical practice (18).
Effective and successful siRNA delivery systems are the Liposome-Polycation-DNA (LPD) particles, developed and characterized by Huang and coworkers, which comprise (carrier) DNA and siRNA complexed with protamine, a strong cationic peptide (26-30) . This overall negatively charged complex is coated with a PEGylated supported double bilayer of cationic lipids to prevent degradation and reticuloendothelial system (RES)-mediated clearance (31). By coupling targeting ligands to the outer surface of these LPD particles, cell specific siRNA delivery upon systemic administration is promoted (26, 29). Since activated angiogenic endothelial cells overexpress $\alpha_v\beta_3$-integrins, they can be specifically targeted with a cyclic Arg-Gly-Asp (RGD) peptide (32-34). By applying a RGD-targeted siRNA delivery vehicle, thereby combining cell-targeted delivery with gene-specific knockdown, a dual-specific system is obtained (35). In the current study, a targeted LPD siRNA delivery system was employed for inhibition of VEGFR-2 expression in angiogenic endothelial cells, as illustrated in Figure 1. RGD-targeted PEGylated LPD particles containing VEGFR-2 siRNA were prepared and optimized for protamine content, DNA content and PEGylation density, and subsequently characterized with regard to their uptake and VEGFR-2 silencing efficacy in murine (H5V) and human (HUVEC) endothelial cells.

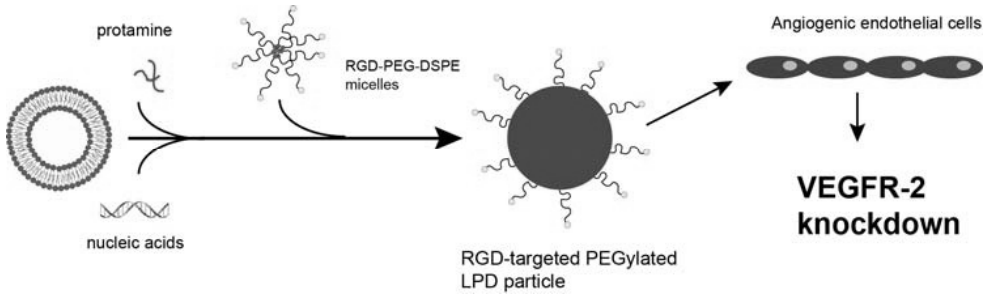

Figure 1. Schematic overview of the preparation of RGD-targeted PEGylated LPD particles for VEGFR-2 knockdown in angiogenic endothelial cells.

MATERIALS & METHODS

Materials

Cholesterol, protamine sulfate salt and calf thymus DNA were purchased from Sigma-Aldrich (St. Louis, USA). *N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propane methylsulphate (DOTAP), methoxy-poly(ethylene glycol)$_{2000}$-distearoylphosphatidylethanolamine (PEG-DSPE) and maleimide–poly(ethylene glycol)$_{2000}$-distearoylphosphatidylethanolamine (mal-PEG-DSPE) were obtained from Avanti Polar Lipids (Alabaster, USA). The cyclic 5-mer RGD c(RGDf[ϵ-S-acetylthioacetyl])K was purchased from JPT Peptide Technologies (Springfield, USA). siRNAs were chemically synthesized and supplied by Eurogentec (Maastricht, The Netherlands). Negative control siRNA was used for gel retardation assay and size and ζ-potential measurements. For uptake studies, the 5′-end of the sense strand was modified with Alexa-488 dye. The sequences of vascular endothelial growth factor receptor-2 (VEGFR-2) siRNAs were 5'-GGA-AAU-CUC-UUG-CAA-GCU-AUU-3'; 5'-UAG-CUU-GCA-AGA-GAU-UUC-CUU-3' targeting the human gene and 5'-CCG-GAA-AUC-UGG-AGA-AUC-ATT-3'; 5'-UGA-UUC-UCC-AGA-UUU-CCG-GTT-3' targeting the murine gene. Antibodies included VEGFR-2 antibody (clone 55B11, Cell Signaling Technology) and β-Actin antibody (clone 13E5, Cell Signaling Technology). Both antibodies were used at a dilution of 1:1000.

Liposome preparation

Liposomes were composed of DOTAP and cholesterol in a 1:1 molar ratio by lipid film hydration. Lipids were dissolved in methanol/chloroform (1:1, v/v) and a lipid film was prepared by rotary evaporation. After drying under nitrogen atmosphere for 45 minutes, the lipid film was hydrated with 5 mM Hepes buffer (pH 7.4). Liposome size was reduced by repeated extrusion through polycarbonate membranes with a final pore size of 50 nm, using a high-pressure extruder (Northern Lipids, Vancouver, British Columbia, Canada).

Preparation of LPD particles

To prepare LPD particles, liposomes and protamine were mixed (final concentrations 8.3 mM total lipid (TL) and 0.2 mg/ml protamine) in ddH_2O and incubated for 10 min. An equal volume of a 1:1 (w/w) mixture of siRNA and calf thymus DNA (0.32 mg/ml) in ddH_2O was added; samples were vortexed and incubated for 10 min. Where indicated, particles were PEGylated by post-insertion using PEG-DSPE or RGD-PEG-DSPE micelles (15 mg/ml based on PEG-DSPE) prepared by lipid film hydration. To prepare RGD-PEG-DSPE micelles, RGD peptide was deacetylated in an aqueous solution of 0.05 M HEPES, 0.05 M hydroxylamine HCl and 25 mM EDTA (pH 7.0) for 30 minutes at

room temperature. Next, the deprotected peptide was incubated overnight at 4°C with the mal-PEG-DSPE micelles. PEG-DSPE or RGD-PEG-DSPE micelles were added to the preformed LPD particles, resuspended, and incubated for 10 minutes at 50 °C.

LPD particle characterization

Mean particle size was measured by Dynamic Light Scattering using an ALV CGS-3 system (Malvern Instruments Ltd., Worcestershire, United Kingdom). DLS results are given as a z-average particle size diameter and a polydispersity index (PDI), which is expressed on a scale of 0 to 1; 0 meaning complete monodispersity and 1 meaning complete polydispersity. ζ- potential measurements were performed using a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom). For agarose gel retardation, nanoparticles containing 20 pmol siRNA were prepared in a final volume of 20 µl. After addition of 4 µl 6x loading dye, samples were loaded on a 4% agarose gel containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed at 100 V for 45 min, after which pictures of the gel were made under UV-illumination.

Cell culture

Human umbilical vein endothelial cells (HUVECs) (Lonza, Verviers, Belgium) were grown in endothelial cell growth medium-2 (EGM-2) (Lonza), consisting of endothelial basal medium-2 (EBM-2) supplemented with a SingleQuots kit (containing growth factors, 2% FBS and antibiotics). Cells were used between passages 3–7. The H5V mouse endothelioma cell line was kindly provided by Dr. G. Molema (Tumor Biology laboratory Groningen) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL Breda) supplemented with 10% FCS, 2 mM glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B).

Cellular uptake studies

Cells (8×10^3 HUVECs or 4×10^3 H5V cells per well) were seeded in 16-well plates on coverslips. After 24 h, cells were transfected with 20 pmol Alexa-488 labeled siRNA complexed in LPD particles. After 4 h of incubation, cells were washed with PBS, fixed with 10% formalin for 30 min, washed again and counterstained with DAPI for 5 min. After a final wash, slides were mounted using Fluorsave (Calbiochem, San Diego, CA, USA) and imaged using a TE2000-U fluorescence microscope TE2000 (Nikon Benelux, Brussels, Belgium).

VEGFR-2 silencing experiments

Cells ($2x10^4$ H5V cells or $4x10^4$ HUVECs per well) were seeded in 12 well plates, 24 h before transfection. Cells were transfected with LPD particles in a total volume of 200 µl (100 pmol siRNA per well for H5V cells and 300 pmol siRNA per well for HUVECs). 4

h after transfection, medium was replaced with complete medium. After 48 h, cells were washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and EDTA for 30 min on ice. Lysates were transferred to eppendorf tubes and centrifuged (14000g, 15 min, 4°C). Supernatants were transferred to new tubes and protein contents were determined using a MicroBCA protein assay (Pierce, Rockford, USA).

To determine VEGFR-2 levels, equal amounts of protein (3-5 µg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes using the iBlot Dry Blotting system (Invitrogen, Paisley, UK). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h at room temperature or overnight (O/N) at 4 °C. After three washes with TBS-T for 5 min, membranes were incubated with primary antibody for 2 h at RT or O/N at 4 °C. Membranes were subsequently washed three times with TBS-T and probed with peroxidase-conjugated secondary antibody for 1 h at RT. After further washing, proteins were visualized with Super Signal West Femto Maximum Sensitivity Substrate Kit (Pierce) and imaged.

RESULTS & DISCUSSION

Preparation and optimization of LPD particles

Particles with a size up to 200 nm have a significantly improved plasma residence time compared to larger particles, as a result of a reduced uptake by the reticuloendothelial system (RES) (36). Additionally, due to increased permeability of the vasculature in tumors, liposomes with a mean diameter of around 100-200 nm accumulate passively in tumors (a phenomenon known as the enhanced permeability and retention (EPR) effect). Liposomes smaller than 70 nm and larger than 300 nm are taken up mainly in liver and spleen, respectively (37-39). LPD particles were prepared with varying ratios of protamine, calf thymus DNA and siRNA, to study the influence on LPD particle size. Firstly, LPD particles were prepared with different amounts of protamine. Protamine is a peptide with high positive charge required for particle condensation. As expected, a higher ratio of protamine to total nucleic acids led to lower mean particle size and polydispersity, indicating an increase in complex condensation (Figure 2A). A protamine/nucleic acid ratio of 0.6 resulted in complexes with mean particle size of approximately 200 nm with low polydispersity. Since higher amounts of protamine did not result in significantly smaller complexes, and too much protamine could change the net charge of the complex to slightly positive - which could interfere with its interaction with cationic lipids (29) - this ratio may be considered optimal for the preparation of LPDs. Secondly, the amount of calf thymus DNA in the particle formulation was optimized, without changing the total amount of nucleic acids (DNA + siRNA). Calf thymus DNA can improve core compaction due to its high molecular weight compared to

siRNA. Whereas DNA alone (DNA/nucleic acid ratio of 1) self-assembled with protamine into nanosized complexes with low polydispersity, siRNA alone (DNA/nucleic acid ratio of 0) did not, illustrating the necessity of (carrier) DNA for the formation of nanoparticles (Figure 2B) (26). A carrier DNA to nucleic acid ratio of 0.5 resulted in complexes with mean particle sizes of approximately 200 nm and low polydispersity, which is suitable for passive targeting using the EPR effect. Since higher amounts of calf thymus DNA did not result in significantly smaller complexes, and the total amount of siRNA in each complex is obviously preferred to be as high as possible, this ratio was considered optimal.

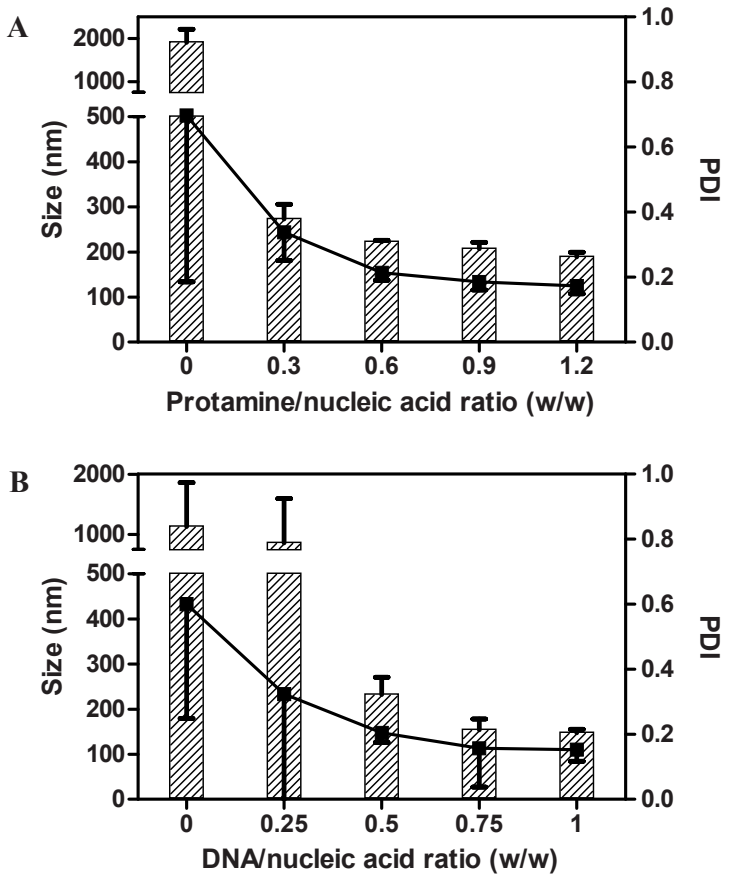

Figure 2. Mean particle size (bars) and PDI (line) of LPD particles prepared with varying ratios of protamine/nucleic acid (with DNA/nucleic acid ratio 0.5) (A) or calf thymus DNA/nucleic acid (with protamine/nucleic acid ratio 0.6) (B). Mean particle size and PDI were determined by dynamic light scattering. Data are presented as average ± SD (n=3).

Steric stabilization of LPD particles by PEGylation

LPD particles are cationic in physiological conditions, independent of the protamine and DNA ratio used (Figure S1). Although a positive charge improves the uptake of liposomes by angiogenic endothelial cells (40), cationic liposomes are susceptible to aggregation upon intravenous injection. This is likely due to interactions with blood proteins, which reduces their *in vivo* circulation time and increases the uptake in liver and spleen (41, 42). Therefore, to improve the colloidal stability of (cationic) liposomes, poly(ethylene glycol) (PEG)-conjugated lipids are commonly used to coat the liposome surface (PEGylation), creating a steric barrier to limit protein adsorption (42-44). In the case of LPD particles, a suitable technique for PEGylation is post-insertion using DSPE-PEG micelles, since PEG-lipids may interfere with the LPD particle formation (45, 46). The influence of PEGylation on complex stability was investigated by a gel retardation assay (Figure 3).

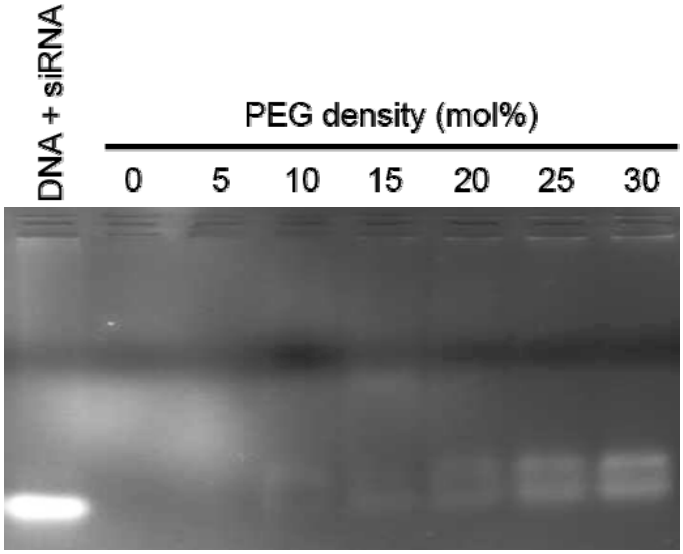

Figure 3. Gel retardation assay of PEGylated LPD particles, prepared with increasing amounts of DSPE-PEG. Samples containing 20 pmol siRNA were applied to a 4% agarose gel containing 0.5 μg/ml ethidium bromide and electrophoresis was performed at 100V for 45 min.

To study the complex stability upon PEGylation, LPD particles containing 0-30 mol% PEG-DSPE were run on an ethidium bromide containing agarose gel to detect the presence of free nucleotides. Up to 15% PEGylation density, no free nucleic acids were detected, indicating stability of the complex within the PEGylated LPD particle. At PEG densities of 20% and higher, PEG-induced release of either siRNA or DNA (or both)

from the LPD particles was observed, illustrated by the higher intensity of the band representing uncomplexed nucleotides at higher PEG densities.
While there was no effect of PEG surface density on the mean LPD particle size and PDI (Figure 4A), the inclusion of PEG-DSPE in the outer bilayer of the cationic LPD particles decreased the ζ-potential with increasing PEG surface density, up to -30 mV in case of 30% PEG (Figure 4B). This effect is probably due to shielding of positive charge in combination with the overall negative charge of PEG-DSPE (47). LPD particles coated with 5%, 10% or 15% PEG show good complex stability and positive surface charge. Whereas 5% PEGylation may be too low to shield the LPD particles from aggregation *in vivo*, and the ζ-potential of LPD particles coated with 15% PEG is close to neutral, LPD particles with 10% surface density of PEG are the most promising for siRNA delivery to angiogenic endothelial cells. These particles can be expected to have good pharmacokinetic properties and to accumulate in tumor tissue as a result of the EPR effect, and were therefore used in further experiments.

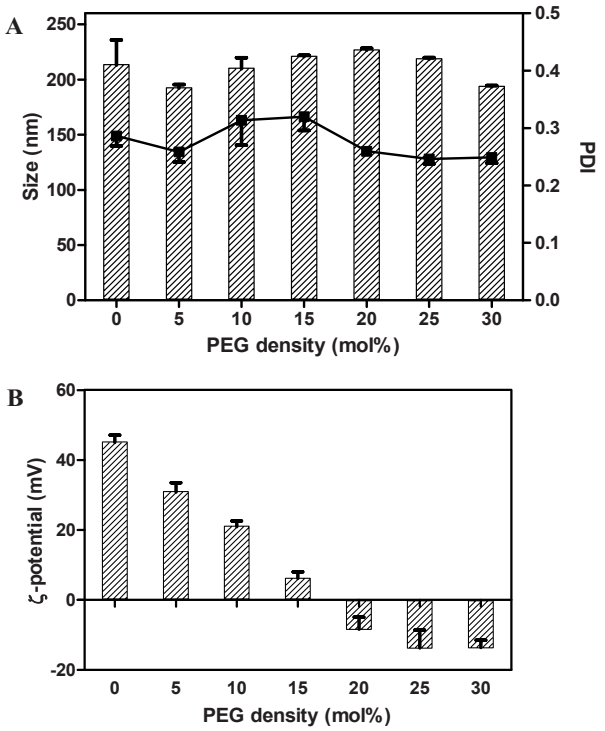

Figure 4. Particle size (bars) and PDI (line) (A) and ζ-potential (B) of PEGylated LPD particles, prepared with increasing amounts of DSPE-PEG. Particle size and ζ-potential were determined by dynamic light scattering and laser Doppler electrophoresis, respectively. Data are presented as mean ± SD (n=3).

RGD-mediated delivery to angiogenic endothelial cells

The small peptide composed of the amino acid sequence arginine, glycine and aspartic acid (RGD) is a well-known targeting ligand for $\alpha_v\beta_3$-integrins expressed by endothelial cells and is internalized upon binding to this receptor (48). To enhance the uptake of the PEGylated LPD particles by angiogenic endothelial cells, PEG-DSPE-conjugated RGD (RGD-PEG-DSPE) was included in the outer bilayer of the LPDs. The RGD peptide density on the surface of the particles was varied by conjugation of RGD to mal-PEG-DSPE micelles in different molar ratios (% RGD) and subsequent incubation of the micelles with the LPD particles for post-insertion. The post-insertion of RGD-PEG-DSPE did not influence the mean particle size and ζ-potential (data not shown).

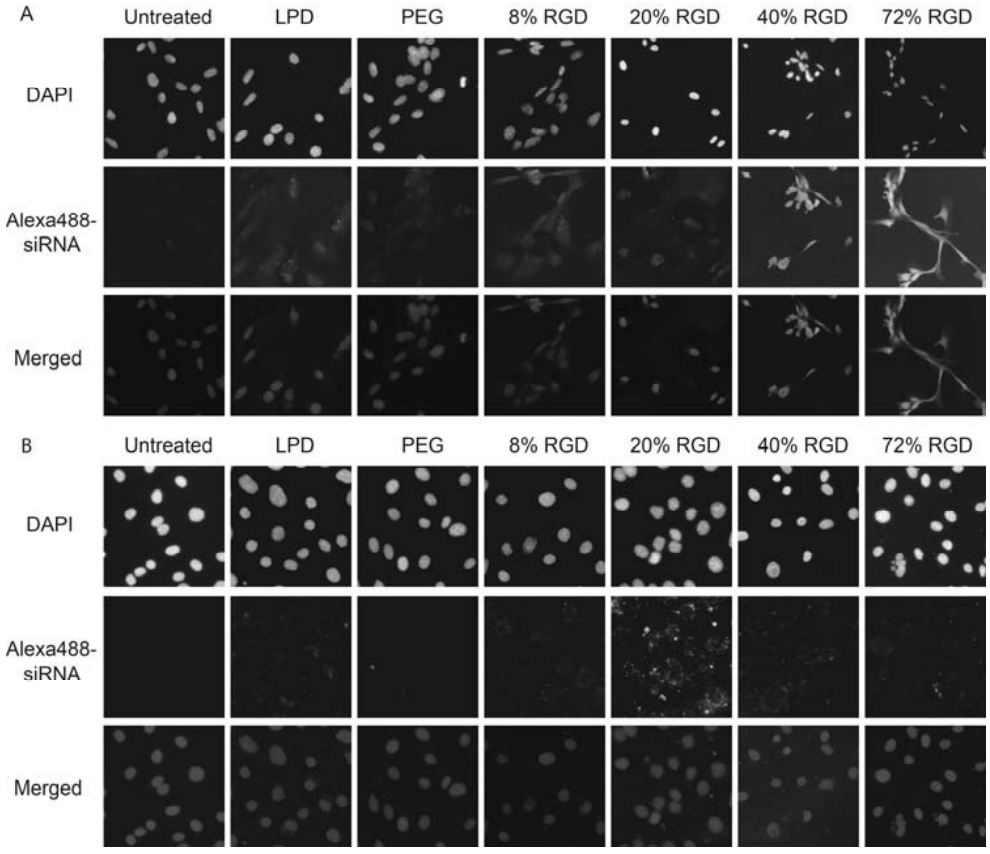

Figure 5. Cellular uptake of Alexa-488 labeled siRNA in various (PEGylated) LPD particles in HUVECs (A) or H5V cells (B). The RGD peptide density on the surface of the particles was varied by reaction of RGD with mal-PEG-DSPE micelles in different molar ratios (% RGD), prior to post-insertion. Images were acquired 4 h after transfection. Nuclei are stained blue (DAPI) and the siRNA appears in green (Alexa488). Images representative of three individual experiments are shown.

The cellular uptake by H5V cells and HUVECs of non-PEGylated LPD particles, PEGylated LPD particles, and PEGylated LPD particles prepared with different quantities of RGD peptide was studied with fluorescence microscopy. After 4 h, non-PEGylated LPD particles were moderately taken up by HUVECs (Figure 5A) and H5V cells (Figure 5B). Whereas hardly any uptake of 10 mol% PEG coated LPDs by both cell lines was observed, RGD-targeting of PEGylated LPD particles enhanced their uptake considerably.

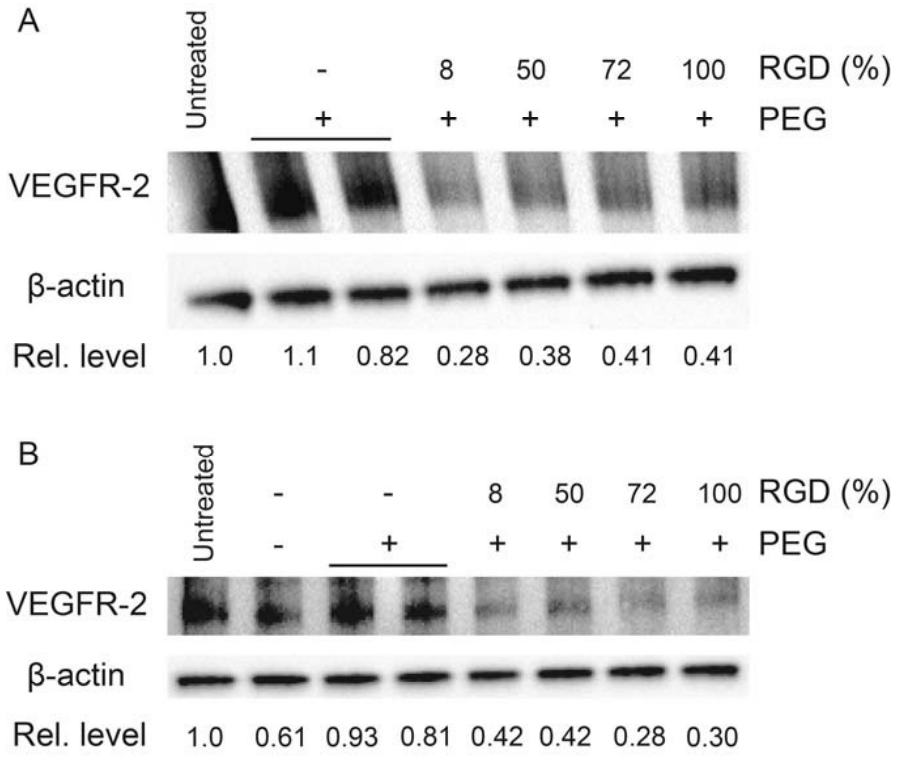

Figure 6. Western Blot analysis of VEGFR-2 expression in HUVECs (A) or H5V cells (B), 48 h after transfection with VEGFR-2 siRNA in various (PEGylated) LPD particles. The RGD peptide density on the surface of the LPD particles was varied by coupling different molar concentrations of RGD (% RGD) with mal-PEG-DSPE micelles, prior to post-insertion. β-actin was used as a loading control.,VEGFR-2 expression levels relative to β -actin are given. Images representative of three individual experiments are shown.

The VEGFR-2 silencing efficacy of RGD-targeted PEGylated LPD particles in HUVECs and H5V cells correlated with the uptake by both cell lines (Figure 6). Non-PEGylated LPD particles were cytotoxic in HUVECs, which prevented assessment of their silencing potential. Nevertheless, these particles induced limited VEGFR-2 silencing in H5V cells

48 h after 4-hour incubation. Although PEGylated LPD particles showed hardly any toxicity on both endothelial cell lines, treated cells expressed VEGFR-2 to a similar extent as untreated cells. In contrast, RGD-targeted PEGylated LPD particles exhibited silencing of VEGFR-2, although no obvious correlation between RGD density and silencing efficacy was observed. In fact, when compared to LPD particles with lower RGD surface densities, 100% RGD-targeted LPD particles appeared to display lower VEGFR-2 silencing efficacy in HUVECs, which may indicate that there is an optimal RGD density on the particle surface. Such an optimum for ligand-density has previously been described for cLABL, a cyclic peptide targeting ICAM-1 on endothelial cells. Higher densities may disturb the receptor clustering behavior upon ligand binding, thereby reducing receptor-ligand affinity (49).

CONCLUSIONS

To accomplish successful targeting of VEGF-mediated angiogenesis by knockdown of VEGFR-2 using RNAi, an efficacious siRNA delivery system is imperative. The optimized RGD-targeted PEGylated LPD particles presented here are promising candidates. They are effectively taken up by murine and human angiogenic endothelial cells and show silencing of VEGFR-2 receptor expression in both cell lines. Therefore, although RNAi is still in an early stage, the use of these RGD-targeted PEGylated LPD particles may assist in bringing angiogenesis-targeted siRNA therapy closer to the clinic.

ACKNOWLEDGEMENTS

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Supporting information

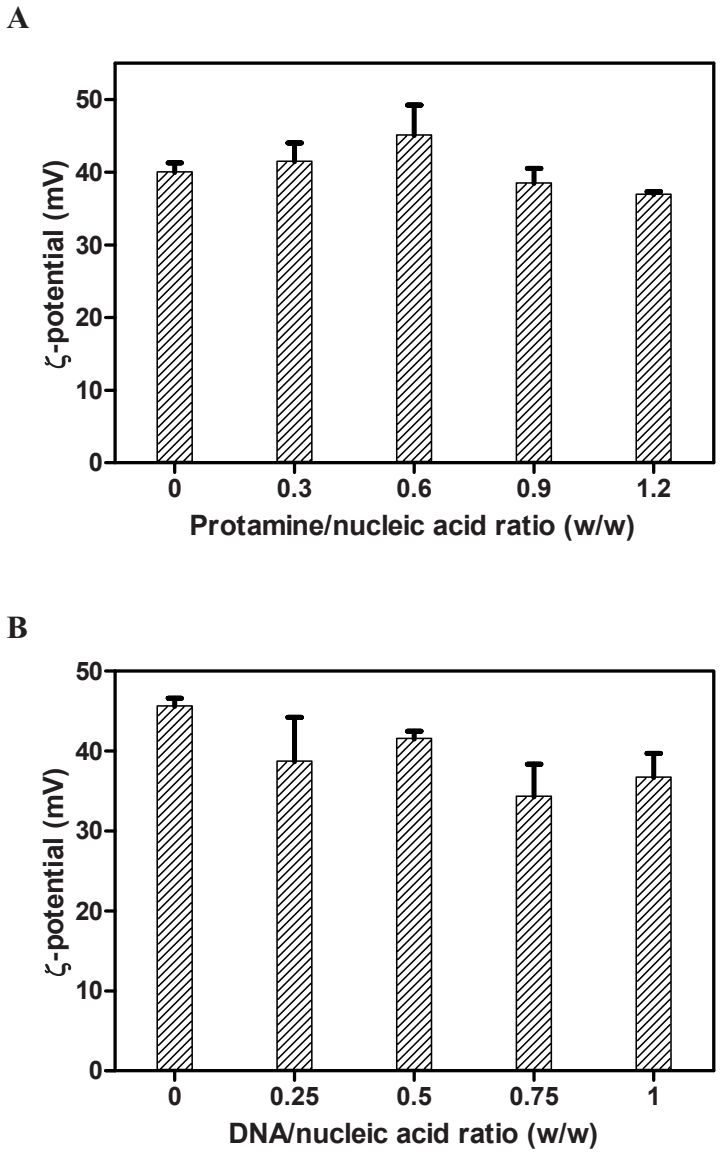

Figure S1. ζ-potential of LPD particles prepared with varying ratios of protamine/nucleic acid (with DNA/nucleic acid ratio 0.5) (A) or calf thymus DNA/nucleic acid (with protamine/nucleic acid ratio 0.6) (B). ζ-potentials were determined by laser Doppler electrophoresis. Data are presented as mean ± SD (n=3).

Chapter 9

Summary and perspectives

INHIBITION OF TUMOR ANGIOGENESIS

In cancer, malignant cells display abnormal and uncontrolled growth in the body. As tumors grow, their oxygen and nutrients consumption and demand increases. When the oxygen tension falls, tumors can induce sprouting of new blood vessels from the pre-existing vasculature, a process called angiogenesis, which is essential for their growth beyond a microscopic size. Arguably, the most critical pro-angiogenic factor is vascular endothelial growth factor (VEGF). VEGF stimulates endothelial cells following binding to integral membrane tyrosine receptor kinases VEGFR-1 and VEGFR-2, which results in activation of multiple intracellular effectors, including the Rho family of small GTPases.

Since 40 years, angiogenesis has been recognized to be a prominent factor in the development of solid tumors and the complex process of neovascularization has since then been regarded as an attractive target for cancer therapy (1, 2). Although several strategies to interfere with the process of angiogenesis have been therapeutically validated in both preclinical and clinical trials, recent studies have shown that current anti-angiogenic therapeutics have severe clinical side effects (3, 4). Therefore, there is a need for novel approaches to specifically target tumor angiogenesis.

A promising strategy to interfere with diseases where (over)expression of specific genes contributes to the pathological process, such as angiogenesis, is gene silencing by RNA interference (RNAi). In **Chapter 2**, we used RNAi to examine the role of the Rho GTPases Rac1 in tumor angiogenesis and growth. Rho GTPases are small molecule members of the Ras superfamily of small GTPases which function as molecular switches in the cell. Rac1 appears to be the major GTPase responsible for VEGF mediated angiogenesis (5) and therefore represents an attractive target for cancer therapy. We used siRNA to specifically silence Rac1 expression in endothelial cells to determine the effect of Rac1 knockdown on angiogenesis *in vitro*. Silencing of Rac1 inhibited VEGF-mediated tube formation, cell migration, invasion and proliferation. In addition, treatment with Rac1 siRNA inhibited angiogenesis in an *in vivo* Matrigel plug assay. Interestingly, intratumoral injections of siRac1 almost completely inhibited the growth of grafted Neuro2a tumors and reduced tumor angiogenesis. Together, these data indicate that Rac1 is an important regulator of VEGF-mediated angiogenesis and that knockdown of Rac1 using siRNA may represent an attractive approach to inhibit tumor angiogenesis and growth. Especially, as several studies have indicated a crucial role for Rac1 in tumor cell migration and invasion, interfering with Rac1 signaling in both tumor- and endothelial cells could improve therapeutic outcome and provide a useful approach towards enduring and effective anti-cancer responses (6). However, as Rac1 is ubiquitously expressed throughout the body and controls a large number of cellular functions, further studies should indicate whether interfering with Rac1 signaling results in undesired side-effects.

Furthermore, progress towards clinical usage of siRNA is still hampered by ineffective delivery into target cells or tissues.

POLY(AMIDO AMINE)S FOR SIRNA DELIVERY

siRNAs are large, negatively charged molecules and are therefore not able to readily cross cellular membranes. Furthermore, they are susceptible to degradation by serum RNAses. For therapeutic application siRNA needs to be protected during circulation and delivered to the desired tissue and into the correct cell type in order to achieve therapeutic effects. Various approaches for the delivery of siRNA have been proposed. Generally two classes of delivery systems can be distinguished, namely viral and non-viral carriers. Non-viral delivery systems, which include cationic polymers and lipids, may circumvent some of the shortcomings of viruses. Although their efficiency is still inferior to their viral counterparts, substantial progress has been made in the last decade to develop and improve these delivery systems. In **Chapter 3**, recent advances in siRNA delivery using polymeric carrier systems are described.

In this thesis, the use of a new class of biodegradable cationic polymers based on poly(amido amine)s with disulfide linkages in the backbone (SS-PAA polymers) for siRNA delivery is described. Due to the difference in redox potential between the oxidizing extracellular space and the reducing intracellular space the disulfide bonds are stable outside the cell, but are rapidly cleaved in the cytoplasm. Previously, SS-PAA copolymers of N,N'-cystaminebisacrylamide (CBA) and 4-amino-1-butanol (ABOL) were shown to self-assemble with plasmid DNA into nano-sized polyplexes and display efficient gene transfer properties (7). In this thesis, these polymers were adapted and further optimized for siRNA delivery.

As described in **Chapter 4**, to be able to compare different polymers on their ability to ensure cellular internalization, a novel technique was developed to quantitatively determine the amount of siRNA inside cells after transfection. For absolute quantification, techniques such as FACS and confocal microscopy, which are widely applied to evaluate cellular uptake, are unreliable, as the results are influenced by quenching phenomena that are dependent on interactions between siRNA and carrier and their intracellular environment. Differences in fluorescent signal of complexed siRNA due to quenching could be overcome by measuring the fluorescent signal after lysing the transfected cells in lysis buffer that contained 2% SDS to dissociate siRNA from the complexes. This method offers a simple approach for quantifying cellular uptake of siRNA, which might help in the development of more efficient delivery systems. However, for carrier systems other than the ones described in this study, the use of a different lysis buffer might be more appropriate, depending on the nature and strength of interactions between siRNA and carrier.

In **Chapter 5**, CBA was copolymerized besides ABOL with 1,2-diaminoethane (EDA) as the amine monomer to introduce more positive charges in the polymer. In this way, copolymers containing different percentages of butanolic side chains and aminoethyl fragments in the main chain were synthesized. Incorporation of EDA in the polymer resulted in increased siRNA condensation. Efficient siRNA condensation was shown to be necessary for cellular uptake, however excess of polymer decreased siRNA uptake for polymers with high amounts of EDA. Silencing efficiency did not correlate with uptake, since silencing increased with increasing w/w ratio for all polymers. More than 80% knockdown was found for polyplexes formed with polymers containing 25% or 50% EDA, which was combined with low cytotoxicity.

Based on the best performing p(CBA-ABOL/EDA) polymer from this study, we synthesized a new PEGylated polymer, p(CBA-ABOL/EDA/PEG), as described in **Chapter 6**. Conjugation of poly(ethylene glycol) (PEG) to cationic polymers (PEGylation) is a common strategy to enhance the biocompatibility of polyplexes. This is necessary, because for cancer therapy, in order to reach distant tumors or metastases, systemic administration of siRNA polyplexes is inevitable. Upon intravenous injection, positively charged polyplexes might potentially interact non-specifically with serum proteins or erythrocytes and other blood cells, leading to formation of aggregates, which causes rapid clearance by the reticulo-endothelial systems (RES) and sometimes significant toxicity (8). In this study, mixtures of the PEGylated and unPEGylated polymer were used in order to vary the PEG content in the final polyplexes. PEGylation effectively decreased polyplex surface charge, salt- or serum-induced aggregation and interaction with erythrocytes. However, increasing the amount of PEG in the formulation also reduced its stability against heparin displacement, cellular uptake and subsequent silencing efficiency. Nevertheless, for polyplexes with high PEG content, significant gene silencing efficacy was found, which was combined with almost complete absence of toxicity.

Encouraged by these results, a pilot study on the influence of polyplex PEGylation on the tissue distribution pattern after intravenous injection was performed in mice, using Alexa-555 labeled siRNA in polyplexes of polymer/siRNA (w/w) ratio 24. However, unexpectedly, administration of all tested formulations, containing 0, 15, 30 or 45 wt% PEG, resulted in systemic toxicity as displayed by decreased motility and respiratory depression, which urged us to sacrifice the mice shortly (within 5-15 minutes) after injection. Post-mortem evaluation revealed macroscopic lung damage, while other organs were unaffected. Interestingly, mice treated with free polymer (with or without PEG) as a control showed similar signs of systemic toxicity, even at half the dose, which suggests that the observed effects might be contributed to the presence of the excess amount of polymer that is present in a free, uncomplexed form in the formulation. The presence and amount of unbound polymer in polyplex formulations have previously been recognized

as inducers of toxicity (9). Therefore, we also injected polyplexes prepared at w/w 12, which contain less free polymer. However, similar toxic effects were observed although the signs of toxicity started at later time points (within 15-30 minutes). As we observed the presence of Alexa-555 siRNA in urine shortly after injection, polyplexes prepared at w/w 12 are apparently not stable in the circulation and after polyplex dissociation, the resulting free polymer possibly again caused toxicity. The remarkable *in vivo* toxicity for these formulations, despite the promising results in our *in vitro* biocompatibility studies pleads for additional *in vitro* assays with higher predictive value.
In addition, it is clear that this formulation still has to be improved in order to enable *in vivo* applications. Further studies should focus on the stabilization of polyplexes which do not contain free polymer. Alternatively, for the current formulation, unbound polymer can be separated from the polyplexes by size exclusion chromatography (SEC) (9) or electrophoresis (10).

LIPIDS FOR SIRNA DELIVERY

As our experiments in mice showed that the current PAA-SS formulation still has to be improved for further *in vivo* application, the possibility of using other non-viral vectors for siRNA delivery was also evaluated in this thesis. Among the wide variety of non-viral vectors, lipid-based carriers have been the most extensively described in both *in vitro* and *in vivo* studies. In **Chapter 7** an overview is provided of currently employed lipid-based delivery systems for siRNA, and of recent research into improvements of cellular delivery by enhancing target cell uptake and redirecting intracellular trafficking routes. Effective and successful siRNA delivery systems are the Liposome-Polycation-DNA (LPD) particles, developed and characterized by Huang and coworkers, which comprise (carrier) DNA and siRNA complexed with protamine, a strong cationic peptide (11-15). In **Chapter 8**, a targeted LPD siRNA delivery system was employed for inhibition of VEGFR-2 expression in angiogenic endothelial cells. Cyclic Arg-Gly-Asp (RGD) peptides, targeting $\alpha_v\beta_3$-integrins which are overexpressed on activated angiogenic endothelial cells, were coupled to the outer surface of these LPD particles. RGD-targeted PEGylated LPD particles containing VEGFR-2 siRNA were prepared and optimized with respect to their size and charge by varying protamine content, carrier DNA content for stronger complexation, and PEGylation density. The uptake and silencing efficacy of the RGD-targeted PEGylated LPD particles were evaluated in H5V cells (murine endothelial cells) and Human Umbilical Vein Endothelial cells (HUVECs). When compared to non-targeted LPD particles, enhanced uptake and silencing of VEGFR-2 expression was observed for RGD-targeted PEGylated LPD particles. Therefore, although still in an early stage, the use of these RGD-targeted PEGylated LPD particles may assist in bringing angiogenesis-targeted siRNA therapy closer to the clinic.

CONCLUSIONS

Angiogenesis is necessary to support the growth of many tumor types and is a well-established target for cancer therapy. Endothelial cells are excellent target cell types for anti-angiogenesis-based therapeutic strategies and targeting the blood vasculature has several advantages as compared to tumor cell targeting, including accessibility and limited development of resistance. However, as current anti-angiogenic therapy only elicits modest effects in clinical settings and shows side effects, there is a strong need for novel strategies to specifically interfere with tumor angiogenesis.

In this thesis, we have employed RNAi to specifically interfere with genes involved in angiogenesis. We have shown that RNAi-mediated silencing of Rac1 can inhibit angiogenesis and tumor growth in mice. Targeting Rac1 is a promising strategy to improve current antitumor therapy. An interesting challenge remains in determining how significant the beneficial effects of targeting Rac1, in combination with conventional anticancer therapies, will be.

Although RNAi holds great promise as an approach to specifically interfere with genes that are difficult to target using conventional strategies, clinical progress is hampered by insufficient delivery of siRNA to the target cell population. We have focused on the development of two different non-viral delivery systems. The disulfide-based poly(amido amine)s described in this thesis are excellent candidates for nucleic acid delivery applications, as they can be easily synthesized and further functionalized to introduce various parameters for optimization for the desired application. Especially the incorporation of disulfide bonds in the polymer chain has been shown to result in enhanced transfection efficiencies and decreased cytotoxicities. By introduction of more positive charges in the polymer, poly(CBA-ABOL/EDA)s were optimized for siRNA delivery. Additionally, PEGylation of these polymers resulted in increased stability of siRNA polyplexes in salt and serum and further decreased their toxicity. Preliminary results *in vivo* indicated that the current formulation still has to be improved in order to enable *in vivo* applications. An important aspect seems to be the reduction of the amount of free polymer in the formulation. The RGD-PEG-LPD systems described in this thesis are promising systems for delivery of siRNA to angiogenic vasculature. We have shown that they can ensure cellular uptake and target gene silencing in both murine and human endothelial cells. Further research should evaluate the use of these carriers for gene silencing *in vivo*.

This thesis provides starting points for future research on the optimization of polymeric and lipidic carrier systems for silencing of genes involved in angiogenesis *in vivo*. This may ultimately lead to the development of safe delivery systems that enable therapeutic RNA interference in cancer therapy.

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Appendices

Nederlandse samenvatting

REMMING VAN TUMOR ANGIOGENESE

Kanker wordt gekenmerkt door de aanwezigheid van kwaadaardige cellen die zich abnormaal en ongecontroleerd vermenigvuldigen in het lichaam. Als tumoren groeien, neemt de vraag naar zuurstof en voedingsstoffen in de tumor toe. Wanneer de zuurstofspanning daalt, kunnen tumoren de vorming van nieuwe bloedvaten vanuit bestaande vaten induceren, een proces dat angiogenese genoemd wordt. Zonder angiogenese kan een beginnende tumor slechts ongeveer een kubieke millimeter groot worden. De belangrijkste factor die de groei van nieuwe bloedvaten bevordert lijkt VEGF te zijn: Vascular Endothelial Growth Factor. VEGF stimuleert endotheelcellen na binding aan de receptoren VEGFR-1 en VEGFR-2, wat leidt tot activatie van verschillende intracellulaire signaalmoleculen, waaronder de Rho familie van kleine GTPases.
Al 40 jaar wordt angiogenese beschouwd als een belangrijke factor in de ontwikkeling van solide tumoren en wordt het ingewikkelde proces van de aanmaak van nieuwe bloedvaten gezien als een aantrekkelijk doelwit voor antikanker therapie. Maar hoewel meerdere strategieën om het angiogenese proces te verstoren al therapeutisch gevalideerd zijn in zowel preklinische als klinische studies, heeft recent onderzoek aangetoond dat de huidige anti-angiogene middelen ernstige bijwerkingen hebben. Om deze reden is er behoefte aan nieuwe manieren om specifiek tumor angiogenese aan te pakken.
Een veelbelovende strategie om in te grijpen in ziektes waarin (over)expressie van specifieke genen bijdraagt aan het pathologische proces, zoals in angiogenese, is het uitschakelen van die genen door middel van RNA interferentie (RNAi), zoals toegelicht in **hoofdstuk 1**. In **hoofdstuk 2** hebben we RNAi gebruikt om de rol van de Rho GTPase Rac1 in tumor angiogenese en tumorgroei te onderzoeken. Rho GTPases zijn onderdeel van de Ras superfamilie van kleine GTPases die werken als moleculaire switches in de cel. Rac1 lijkt de belangrijkste GTPase te zijn in VEGF-gemedieerde angiogenese en zou daarom een aantrekkelijk doelwit in antikanker therapie kunnen zijn. We hebben siRNA gebruikt om specifiek expressie van Rac1 in endotheelcellen te remmen en daarmee het effect van Rac1 ‘knockdown’ op angiogenese *in vitro* vast te stellen. Het remmen van Rac1 expressie resulteerde in een verminderde VEGF-gemedieerde formatie van cellulaire uitlopers, cel migratie, invasie en proliferatie. Daarnaast remde behandeling met Rac1 siRNA angiogenese in een *in vivo* Matrigel plug assay. Interessant genoeg remden intratumorale injecties van siRac1 de angiogenese en groei van onderhuidse neuroblastoma tumoren bijna volledig. Collectief tonen deze data aan dat Rac1 een belangrijke rol in VEGF-gemedieerde angiogenese speelt en dat remming van Rac1 expressie met behulp van siRNA een aantrekkelijke mogelijkheid zou kunnen zijn om tumor angiogenese en groei tegen te gaan. Bovendien, aangezien in meerdere studies ook een cruciale rol voor Rac1 in tumorcel migratie en proliferatie is aangetoond, zou het tegelijkertijd verstoren van Rac1 signalering in tumor- en endotheelcellen het

therapeutisch effect kunnen versterken en daarmee een effectieve en langdurende antikanker respons dichterbij brengen. Maar, aangezien Rac1 door het hele lichaam tot expressie wordt gebracht en een rol speelt in vele processen in de cel, zal verder onderzoek eerst moeten uitwijzen of het verstoren van Rac1 signalering tot ongewenste bijwerkingen leidt. Bovendien worden vorderingen richting gebruik van siRNA in de kliniek nog steeds tegengehouden doordat siRNA niet goed kan worden afgeleverd in de juiste cellen of het juiste weefsel. Ook dit aspect is in dit promotie-onderzoek onderzocht.

POLY(AMIDO AMINE)S VOOR HET AFLEVEREN VAN SIRNA

siRNAs zijn grote, negatief geladen moleculen en kunnen daardoor niet zomaar celmembranen passeren. Bovendien zijn ze gevoelig voor afbraak door serum RNAses. Voor therapeutische toepassingen moet het siRNA beschermd worden in de circulatie en afgeleverd in het gewenste weefsel en in het juiste celtype om effect te hebben. Verscheidene manieren voor het afleveren van siRNA zijn inmiddels voorgesteld. Grofweg kunnen daarin twee klassen van afleversystemen worden onderscheiden, namelijk virale en niet-virale systemen. Niet-virale afleversystemen, waaronder katione polymeren en lipiden, zouden sommige van de tekortkomingen van virussen kunnen vermijden, zoals veiligheidrisico's en problemen in de productie. En hoewel ze nog steeds minder efficiënt zijn dan virussen, zijn er in het afgelopen decennium behoorlijke vorderingen gemaakt in de ontwikkeling en verbetering van deze afleversystemen. In **hoofdstuk 3** worden de meeste recente ontwikkelingen op het gebied van siRNA aflevering door polymeersystemen bediscussieerd.

In dit proefschrift is het gebruik beschreven van een nieuwe klasse van biodegradeerbare katione polymeren gebaseerd op poly(amido amine)s met disulfide linkers in de 'ruggengraat' (SS-PAA polymeren) voor het afleveren van siRNA. Door het verschil in redox potentiaal tussen de oxiderende extracellulaire ruimte en de reducerende intracellulaire ruimte zijn de disulfide linkers stabiel buiten de cel, maar vallen ze uiteen in het cytoplasma. Eerder werd al aangetoond dat SS-PAA copolymeren van N,N'-cystaminebisacrylamide (CBA) en 4-amino-1-butanol (ABOL) vanzelf nano-deeltjes (polyplexen) vormen met plasmide DNA en goede eigenschappen voor gentransfectie bezitten.

Zoals beschreven in **hoofdstuk 4** hebben we eerst een nieuwe methode ontwikkeld om kwantitatief siRNA in cellen na transfectie te bepalen, om verschillende polymeren te kunnen vergelijken op hun vermogen om siRNA in cellen af te leveren. Voor absolute kwantificatie zijn technieken zoals FACS en confocale microscopie, onbetrouwbaar, omdat de resultaten beïnvloed worden door 'quenching' processen die afhankelijk zijn van de interacties tussen siRNA en dragersysteem en het intracellulaire milieu. Desalniettemin worden ze veelvuldig toegepast. Verschillen in het fluorescentie-signaal

van gecomplexeerd siRNA als gevolg van quenching konden worden hersteld door het signaal te meten nadat de getransfecteerde cellen gelyseerd waren in een lyseerbuffer die 2% SDS bevatte om het siRNA uit de complexen te vrij te maken. Deze methode biedt een simpele manier om opname van siRNA in cellen te kwantificeren, wat kan helpen bij het ontwikkelen van efficiëntere afleversystemen. Voor andere dragersystemen dan die beschreven in deze studie is het echter mogelijk dat een andere lysisbuffer geschikter is, afhankelijk van de wijze en sterkte van de interactie tussen siRNA en dragersysteem.

In **hoofdstuk 5** hebben we CBA gecopolymeriseerd met, naast ABOL, 1,2-diaminoethane (EDA) als amine monomeer om meer positieve ladingen in het polymeer te introduceren. Op deze manier werden copolymeren gesynthetiseerd met verschillende percentages aan butanol zijketens en aminoethyl fragmenten in de hoofdketen. Incorporatie van EDA in het polymeer resulteerde in versterkte siRNA condensatie. Voldoende siRNA condensatie was noodzakelijk voor opname in cellen, hoewel een overmaat aan polymeer met grote hoeveelheden EDA siRNA opname weer hinderde. De gen-uitschakeling efficiëntie correleerde niet met de opname, aangezien de mate van gen-uitschakeling toenam met een toenemende gewichtsratio voor alle polymeren. Er werd meer dan 80% gen-uitschakeling gevonden bij polyplexen die waren gevormd met polymeren die 25% of 50% EDA bevatten. Deze lieten bovendien een lage cytotoxiciteit zien.

Gebaseerd op het meest veelbelovende p(CBA-ABOL/EDA) polymeer van deze studie werd, zoals beschreven in **hoofdstuk 6**, een nieuw, gePEGyleerd polymeer gesynthetiseerd, p(CBA-ABOL/EDA/PEG). Het conjugeren van poly(ethyleen glycol) (PEG) aan katione polymeren (PEGylering) is een veelgebruikte strategie om de biocompatibiliteit van polyplexen te verbeteren. Dit is noodzakelijk in antikanker therapie omdat, om de tumor of metastase te bereiken, systemische toediening van siRNA polyplexen vaak onvermijdelijk is. Na intraveneuze toediening kunnen positief geladen polyplexen mogelijk een niet-specifieke interactie met serum eiwitten of bloedcellen aangaan, wat leidt tot de formatie van aggregaten die snel geklaard worden door het reticulo-endotheel systeem (RES) maar ook behoorlijke toxiciteit tot gevolg kunnen hebben. In deze studie werden mengsels van gePEGyleerd en ongePEGyleerd polymeer gebruikt om de hoeveelheid PEG in de uiteindelijke polyplexen te variëren. PEGylering van polyplexen resulteerde in afname van de oppervlaktelading, van zout- of serumgeïnduceerde aggregatie en van interactie met erythrocyten. Een toenemende hoeveelheid PEG in de formulering leidde echter ook tot een verminderde stabiliteit in aanwezigheid van heparine als concurrerend polyanion, verminderde opname in cellen en daarmee ook een lagere gen-uitschakeling efficiëntie. Desalniettemin werd bij polyplexen met een grote hoeveelheid PEG significante gen-uitschakeling gevonden, bijna geheel zonder toxiciteit.

LIPIDEN VOOR HET AFLEVEREN VAN SIRNA

Tegelijkertijd hebben we in dit proefschrift ook de mogelijkheid bekeken om andere niet-virale systemen te gebruiken voor het afleveren van siRNA. Onder de grote verscheidenheid aan niet-virale vectoren zijn de lipide-gebaseerde systemen het uitgebreidst beschreven in zowel *in vitro* als *in vivo* studies. In **hoofdstuk 7** wordt een overzicht gegeven van de op dit moment gebruikte lipide-gebaseerde afleversystemen voor siRNA, en van recent onderzoek naar verbeteringen in de aflevering door opname in de doelwitcellen te vergroten en de intracellulaire transportroute te sturen.
Effectieve en succesvolle siRNA afleversystemen zijn de Liposoom-Polykation-DNA (LPD) deeltjes, ontwikkeld en gekarakteriseerd door Huang en collega's. Deze systemen bevatten (drager) DNA en siRNA gecomplexeerd met protamine, een sterk kationisch peptide. In **hoofdstuk 8**, hebben we een gericht LPD siRNA afleversysteem gebruikt om VEGFR-2 expressie in angiogene endotheelcellen te remmen. Daartoe werden cyclische Arg-Gly-Asp (RGD) peptiden, die specifiek aan $\alpha_v\beta_3$-integrines die tot overexpressie komen op geactiveerde endotheelcellen kunnen binden, aan de buitenkant van deze LPD deeltjes gekoppeld. De RGD-gekoppelde, gePEGyleerde LPD deeltjes werden gemaakt met VEGFR-2 siRNA en geoptimaliseerd met betrekking tot hun grootte en lading, door de hoeveelheid protamine, drager DNA (voor sterkere complexatie) en PEG te variëren. De opname en gen-uitschakeling efficiëntie van deze systemen werd bestudeerd in H5V cellen (muis endotheelcellen) and HUVECs (humane endotheel cellen). In vergelijking tot de niet-gerichte LPD systemen werd een verbeterde opname en remming van VEGFR-2 expressie gevonden bij de RGD-gekoppelde, gePEGyleerde LPD deeltjes. Hoewel de ontwikkeling nog in een vroeg stadium is, zou het gebruik van deze deeltjes mogelijk kunnen helpen om angiogenese-gerichte siRNA therapie dichterbij de kliniek te brengen.

CONCLUSIES

Angiogenese is noodzakelijk voor de groei van vele typen tumoren en is daarmee een erkend doelwit in antikanker therapie. Endotheelcellen zijn uitstekende doelwitcellen voor anti-antiogenese-gebaseerde therapeutische strategieën en het richten op de bloedvaten heeft meerdere voordelen ten opzichte van het richten op de tumorcellen zelf, zoals de toegankelijkheid en verminderde ontwikkeling van resistentie. Omdat huidige anti-angiogene therapie echter slechts bescheiden effecten laat zien in een klinische setting en bovendien bijwerkingen heeft, is er grote behoefte aan nieuwe manieren om specifiek in te grijpen in tumor angiogenese.
In dit proefschrift hebben we RNAi gebruikt om specifiek genen uit te schakelen die betrokken zijn bij angiogenese. We hebben laten zien dat uitschakeling van Rac1

angiogenese en tumorgroei kan remmen in muizen. Dit lijkt een veelbelovende strategie om huidige antikanker therapie te verbeteren. Een interessant uitdaging blijft echter om vast te stellen hoe significant de effecten van Rac1 uitschakeling zijn in combinatie met conventionele antikanker therapie.

Hoewel RNAi veelbelovend is als een manier om specifiek genen uit te schakelen die moeilijk aan te pakken zijn met conventionele strategieën, wordt vooruitgang in de kliniek geremd door onvoldoende aflevering van siRNA naar de juiste celpopulatie. Wij hebben ons geconcentreerd op de ontwikkeling van twee verschillende niet-virale afleversystemen. De disulfide-gebaseerde poly(amido amine)s die zijn beschreven in dit proefschrift zijn uitstekende kandidaten voor het afleveren van nucleïnezuren, omdat ze gemakkelijk gesynthetiseerd en verder gefunctionaliseerd kunnen worden om daarmee verschillende parameters voor optimalisatie voor de gewenste toepassing toe te voegen. Met name de toevoeging van disulfide bruggen in de polymeerketen heeft geresulteerd in verhoogde transfectie efficiëntie en verlaagde cytotoxiciteit. Door meer positieve ladingen in het polymeer te introduceren werden poly(CBA-ABOL/EDA)s nu geoptimaliseerd voor het afleveren van siRNA. Daarnaast leidde PEGylering van deze polymeren tot meer stabiliteit van de siRNA polyplexen in aanwezigheid van zout en serum en tot verminderde toxiciteit. De RGD-PEG-LPD systemen die zijn beschreven in dit proefschrift zijn ook veelbelovende systemen voor het afleveren van siRNA in angiogene bloedvaten. We hebben laten zien dat ze opname in cellen en gen-uitschakeling kunnen bewerkstelligen in zowel muis als humane endotheelcellen. Verder onderzoek zal het gebruik van deze systemen voor gen-uitschakeling *in vivo* moeten evalueren.

Dit proefschrift levert aanknopingspunten voor verder onderzoek naar de optimalisatie van polymere en lipide dragersystemen voor het uitschakelen van genen die betrokken zijn bij angiogenese *in vivo*. Dit kan uiteindelijk leiden tot de ontwikkeling van veilige afleversystemen die het gebruik van RNAi in antikanker therapie mogelijk maken.

Curriculum Vitae &
List of publications

Pieter Vader was born on April 25th 1985 in Utrecht, The Netherlands. In 2002, he graduated from the Christelijk Gymnasium in Utrecht, after which he started his Bachelor's program in Chemistry at Utrecht University. In 2005, he started a Master's program in Drug Innovation at Utrecht University. During this Master's he performed a 9-month research project in the department of Pharmacology, at Utrecht University, focusing on the effects of cigarette smoke on the inflammatory response of neutrophils. This project included a 2-month stay at the National Heart and Lung Institute at Imperial College, London, where he studied the influence of HDAC proteins in the neutrophil response. After this, he performed a 6-month internship in the department of Pharmaceutical & Analytical Development at Solvay Pharmaceuticals B.V. (now Abbott Healthcare Products B.V.) in Weesp, to work on the development of in vitro models for dissolution of drugs in the gastrointestinal tract. After obtaining his Master's degree, he started his PhD project in the department of Pharmaceutics at Utrecht University on targeted delivery of siRNA to inhibit tumor angiogenesis under supervision of prof. dr. Gert Storm and dr. Raymond M. Schiffelers, in close collaboration with the University of Twente. The results of this project are presented in this thesis. Currently, he works as a postdoctoral researcher in the department of Clinical Chemistry and Hematology at the University Medical Center Utrecht.

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"O LORD, how manifold are Your works!
In wisdom You have made them all.
The earth is full of Your possessions."

Psalm 104:24
New King James Version (NKJV)