

Chapter 2

Sequence-Defined Oligoaminoamides for the Delivery of siRNAs

Dian-Jang Lee, Ernst Wagner, and Taavi Lehto

Abstract

Since it was found that synthetic small interfering RNA (siRNA) can invoke RNA interference (RNAi) responses in mammalian cells, it has gained enormous attention as a tool for gene silencing in basic science and as a novel therapeutic modality. To develop carriers for cytosolic and systemic siRNA delivery, our laboratory has recently developed a sequence-defined polymer platform compatible with solid-phase-supported synthesis. These polymers have displayed efficient siRNA-mediated gene silencing in vitro and in vivo. In this chapter, we provide a brief background on the special features of these polymers and detailed protocols to evaluate polyplex formation, gene silencing efficiency, and cytotoxicity of siRNA-containing polyplexes.

Key words siRNA delivery, Gene silencing, Sequence-defined polymer, Polyplex, Nanoparticle, Polyaminoamide, Oligoaminoamide

1 Introduction

RNA interference (RNAi) has been one of the most groundbreaking discoveries of the past 15 years in the field of molecular biology. RNAi is an evolutionarily conserved phenomenon for sequence-specific gene silencing among multicellular organisms as diverse as plants, worms, yeast and humans, in which double-stranded RNA triggers specific degradation of the complementary mRNA sequence to silence the expression of a target gene [1]. Since it was found that synthetic small interfering RNA (siRNA) can also invoke RNAi responses in mammalian cells [2], this novel strategy has rapidly become a powerful tool for sequence-specific gene silencing, with many lead compounds in various stages of clinical development [3]. As viral vectors are not compatible with the delivery of synthetic siRNAs, different nonviral delivery systems have sparked intense investigations [4].

The main hurdles against efficient cytosolic siRNA delivery include: (i) high charge distribution and size of siRNAs impede

their translocation through the cell membrane [5]; (ii) siRNAs are biologically fragile and are actively targeted by nucleases for degradation in extracellular and intracellular environments [6]; (iii) upon systemic administration, negative charge of siRNA may be recognized by pattern recognition receptors (PRRs) triggering the innate immune response [4]; and (iv) naked siRNAs are subject to rapid elimination by renal clearance [7]. It is generally realized that the key challenges for transforming siRNAs from valuable research tool to clinical applications are highly dependent on the development of safe and efficient delivery systems.

As aforementioned, different nonviral vectors have been successfully used for the delivery of siRNAs, both in vitro and in vivo [5, 8]. Recently, our laboratory has developed a solid-phase-supported synthesis method [9], where polyaminoamide building blocks (as proton-sponge motifs) were applied together with lysines (as branching units), various fatty acids (as stabilizing hydrophobic domains), and cysteines (as bioreversible disulfide-forming units) to generate sequence-defined monodisperse peptide-like polymers [10–12].

It is widely recognized that nonviral vectors utilize different endocytic pathways in order to gain access to the cells [13]. Consequently, both vectors and their respective cargo remain to high extent entrapped in endosomal compartments and this endosomal entrapment serves as the main limiting factors in their efficient delivery. Many different strategies have been investigated aiming to induce endosomal escape [14]. Above-mentioned polyaminoamide-based building blocks (e.g. Ptp, Stp, or Gtp) (Fig. 1a) are designed in the way that they would act as proton-sponge motifs, which express about 20 % protonation of nitrogens at neutral pH and would become increasingly protonated with endo-lysosomal acidification. The influx of protons is followed by counterions and water, increase of osmotic pressure, leading to the subsequent rupture of the endosomal membrane and release of the polyplexes [10, 14].

It is believed that polyplex formation between the cationic carriers and nucleic acids, i.e. siRNA, takes place due to the electrostatic interaction between the nitrogens of the polymers and the phosphates of the siRNAs [15]. In addition to electrostatic interaction, hydrogen bonding and hydrophobic interactions contribute to the formation and stability of polyplexes [14]. In our sequence-defined polymers, we used different strategies to increase particle stability and endosomal escape. For example, lipid moieties (Fig. 1b) can be incorporated to increase hydrophobicity, improve polyplex stability, and also introduce pH-dependent lytic activity that leads to increased endosomal escape [9, 10]. Moreover, cysteines can be introduced to allow cross-linking through disulfide bridge formation and thereby increase particle stability (Fig. 1c) [10, 16].

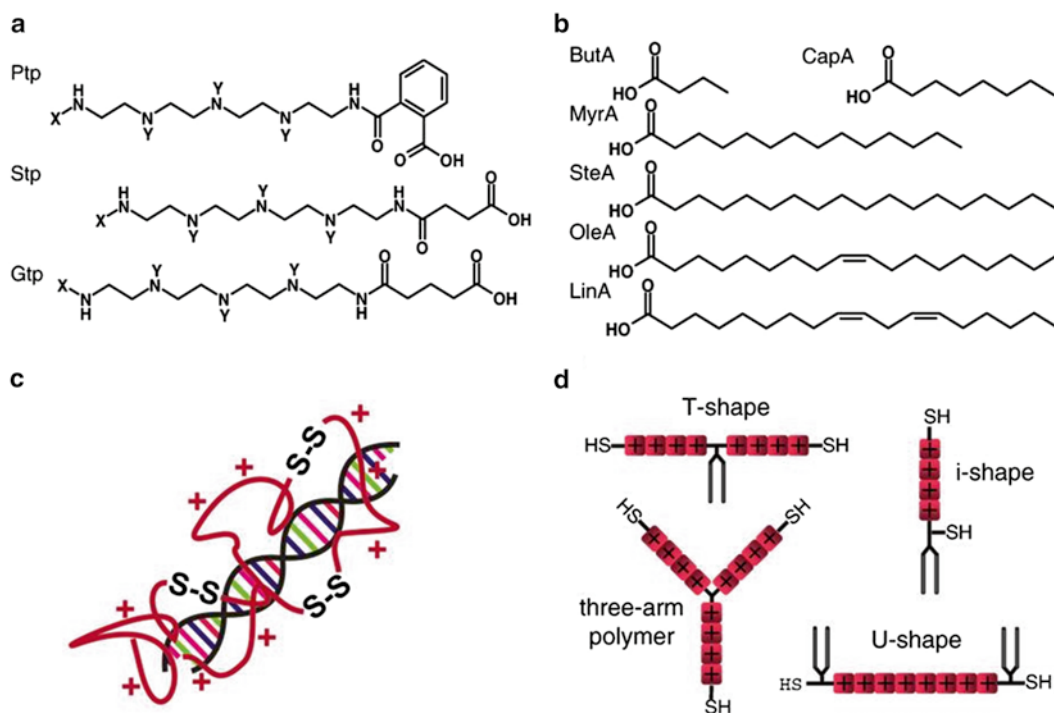


Fig. 1 Strategies for polymer design. **(a)** Artificial amino acids Ptp, Stp or Gtp with protective groups during synthesis ($X = \text{Fmoc}$, $Y = \text{Boc}$) or without ($X = Y = \text{H}$) after synthesis is complete. These building blocks contain the same proton-sponge 1,2-diaminoethane motif as present in polyethylenimine [16]. **(b)** Fatty acids butyric acid (ButA), caprylic acid (CapA), myristic acid (MyrA), stearic acid (SteA), oleic acid (OleA) or linolic acid (LinA) are used for hydrophobic modification of polymers. **(c)** Disulfide cross-linking of cysteines during polyplex formation. **(d)** Different topologies of the polymers: branched three-armed structure with a lysine as branching point; *i*-shape with two fatty acids on one side of the backbone; *U*-shape with two fatty acids on each end of the backbone; *T*-shape with two fatty acids incorporated in the middle of the polymer backbone. “+” represents a cationic oligoamino acid building block (Ptp, Stp, Gtp) (reproduced from ref. [10] with permission from Elsevier)

In this case, it has been hypothesized that siRNA might act as a template, bringing the positively charged polymer molecules together into close distance, and thus accelerating disulfide formation. It is also important that disulfide bonds are bioreversible, which are stable in the extracellular environment, i.e. systemic circulation, but readily cleaved in the reducing milieu of the cytosol [14]. Furthermore, these polymers could be synthesized with very diverse molecular topologies, for example *i*-shape, *T*-shape, *U*-shape or branched three-armed configurations (Fig. 1d), and more. Promising candidates of the library such as polymers 49, 229, 278, 386 and 454 (Fig. 2) have shown high activity for siRNA delivery, enabling successful silencing of target genes of interest in both in vitro and in vivo settings [9–12]. The addition of oligotyrosines

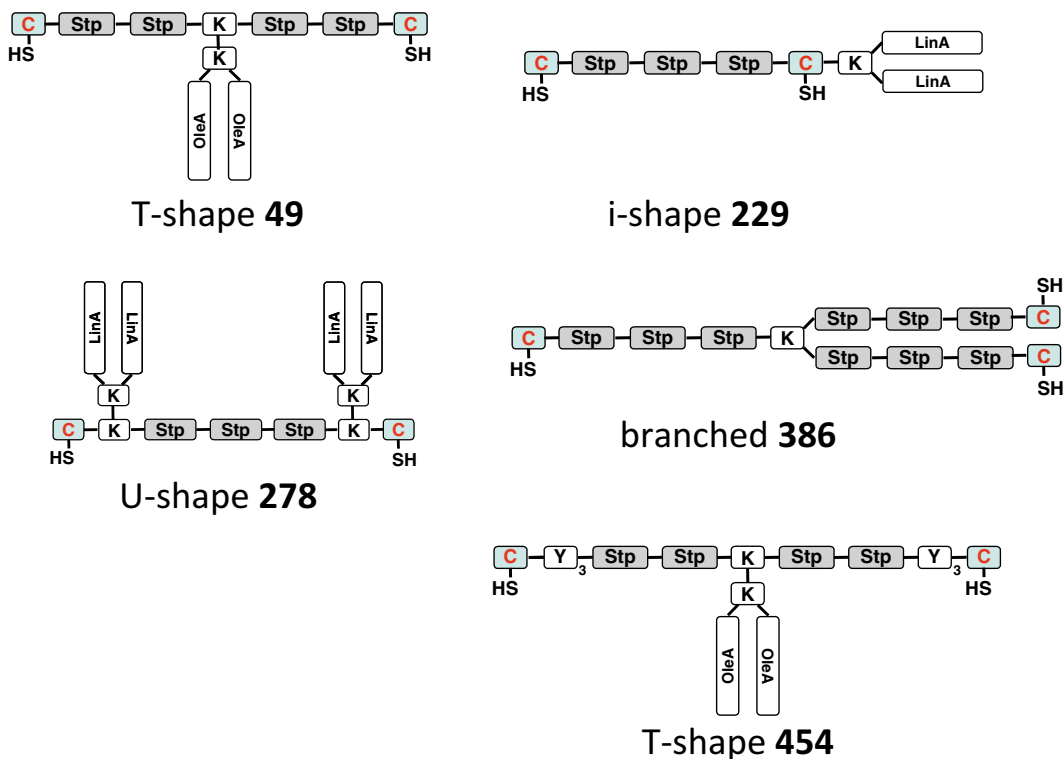


Fig. 2 Structure of polymers **49**, **229**, **278**, **386**, and **454**. These polymers are modified with OleA or LinA lipid moieties (with the exemption of **386**) and contain terminal cysteines (C) which form intermolecular disulfide bonds during siRNA polyplex formation. In case of polymer **454**, tyrosine tripeptides (Y₃) are added next to the terminal cysteines as additional stabilizing components for siRNA polyplex formation (reproduced from ref. [10] with permission from Elsevier)

to the polymer **454** renders the vector even more potent in terms of forming stable polyplexes and gene silencing efficiency [11]. From the perspective of systemic delivery, polymer **386** has displayed the fastest onset of protein knockdown and highest in vivo antitumoral effect [12]. These polyamidoamine-based defined polymers are also flexible for further functionalizing modifications, as targeting (e.g. folic acid) and shielding moieties (e.g. polyethylene glycols) have been successfully incorporated into such polymers [7].

In this chapter, we describe a series of assays that could be used to successfully screen for efficient siRNA nanocarriers. At first, we show how agarose gel shift assay could be utilized to assign the polyplex formation between polymers and siRNA. Secondly, we describe how reporter cell lines could be used to evaluate gene silencing potential of siRNA polyplexes. Finally, we show how cell viability assay could be employed to monitor cytotoxic side-effects.

2 Materials

2.1 Polyplex Formation

1. Synthesis and purification of polymers **49**, **229**, **278**, **386**, and **454** are described in refs. [9–11]. For in vitro tests, we dissolve polymers with RNase-free Millipore water and store 5 mg/ml in 50 µl of aliquots at –20 °C.
2. siRNA against eGFP^{Luc} fusion protein (siGFP) and control siRNA (siControl) synthesized by Axolabs GmbH, Germany. The sequences are shown in Table 1. We prepare 0.5 µg/µl in 50 µl of aliquots (dissolved in RNase-free Millipore water) at –20 °C (*see* **Note 1**).
3. 20 mM HEPES (Biomol, Germany) buffered 5 % glucose (Merck, Germany) pH 7.4 (HBG), in 5 ml of aliquots at –20 °C.
4. 1.5 ml Eppendorf tubes (Brand, Germany).

2.2 siRNA Binding Assay

1. Agarose powder (Invitrogen, Germany).
2. 1× TBE (Tris-borate-EDTA) Buffer (Sigma, Germany).
3. GelRed® (Biotium, USA).
4. Bromophenol blue. Store at 4 °C.
5. Electrophoresis system. The well comb should be large enough to form 25 µl of gel pockets, e.g. Sub-Cell GT System (Bio-Rad, USA).
6. UV transilluminator, e.g. Dark Hood DH-40 (Biostep, Germany).

2.3 Cell Culture

1. Cell lines stably transfected with the eGFP-luciferase gene are used for siRNA silencing assay, and wild-type cell lines are used for cell viability assay. The cell lines and corresponding culture media are described in Table 2.
2. Trypsin (0.25 %) and ethylenediamine tetraacetic acid (EDTA) (1 mM) (Invitrogen, Germany).
3. Phosphate buffered saline (PBS) (Invitrogen, Germany).
4. 75 cm² cell culture flasks (TPP, Switzerland).
5. 96-well cell culture plates (TPP, Switzerland).

Table 1
Oligonucleotides used

siRNA	Target	Supplier	siRNA sequence
siGFP	GFP	Axolabs	AuAucAuGGccGAcAAGcAdTsdT (Sense) UGCUUGUCGGCcAUGAuAUdTsdT (Antisense)
siControl		Axolabs	AuGuAuuGGccuGuAuuAGdTsdT (Sense) CuAAuAcAGGCcAAuAcAUdTsdT (Antisense)

Modified nucleotides: small letters, 2'methoxy; dT, deoxythymidine; s, phosphorothioate linkage

Table 2
Cell lines and corresponding culture media used here

Cell line	Cell culture media
Mouse neuroblastoma Neuro2A/eGFP _{Luc} cells	Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents purchased from Invitrogen, Germany
Human hepatoma HepG2/ eGFP _{Luc} cells	Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents purchased from Invitrogen, Germany
Human prostate carcinoma DU-145/eGFP _{Luc} cells	RPMI-1640 medium, 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents purchased from Invitrogen, Germany
Mouse neuroblastoma Neuro2A cells	Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents purchased from Invitrogen, Germany
Human hepatoma HepG2 cells	Dulbecco's modified Eagle's medium (DMEM), 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents purchased from Invitrogen, Germany
Human prostate carcinoma DU-145 cells	RPMI-1640 medium, 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All reagents purchased from Invitrogen, Germany

2.4 siRNA Silencing Assay

1. Cell culture 5× lysis reagent (Promega, Germany).
2. 10 mM luciferin solution: Weigh and add 48 mg of luciferin (Promega, Germany) to 0.47 ml of 1 M glycylglycine (pH 8.0) (Sigma, Germany) and 10 ml of Millipore water. Then adjust with 1 M NaOH (AppliChem, Germany) to pH 8.0. Finally, bring to 16 ml with Millipore water and freeze in 500 µl of aliquots. Store below −20 °C.
3. Luciferase assay reagent (LAR): Weigh and add 50.8 mg of DTT (Sigma, Germany) and 27.8 mg of ATP (Roche, Germany) to 2 ml of 1 M glycylglycine (Sigma, Germany), 1 ml of 100 mM MgCl₂ (Sigma, Germany), 20 µl of 500 mM EDTA (Sigma, Germany), and 0.5 ml of 42.6 mg/ml coenzyme A (Sigma, Germany). Fill up to 100 ml with Millipore water. Adjust with 1 M NaOH (AppliChem, Germany) to pH 8–8.5. Store at 4 °C (*see Note 2*).
4. Luminometer, e.g. Centro LB 960 plate reader luminometer (Berthold, Germany).
5. Microplate 96-well, white (Berthold, Germany). The multi-well plates must be compatible with the luminometer used.

2.5 Cell Viability Assay

1. CellTiter-Glo® Reagent (Promega, Germany). Store at -20°C .
2. White 96-well plates (Nunc, Germany). The multiwell plates must be compatible with the luminometer used.
3. Orbital shaker, e.g. Duomax 1030 (Heidolph, Germany).
4. Luminometer, e.g. Lumat LB 960 (Berthold, Germany).

3 Methods

In order to find optimal conditions for particle formation it is necessary to screen different N/P (pronatable amines of the polymer/phosphates of the siRNA) ratios, especially as the properties of polymers (amount of nitrogen, etc.) vary greatly. The simplest method for screening of polyplex formation (binding of polymers to siRNA) is a gel-shift assay on agarose gel. The negatively charged naked siRNA is small enough to diffuse throughout the gel in electrical field, whereas when polymer start to bind to the siRNA it retards the siRNA diffusion and therefore indirectly allows the evaluation of polyplex formation. The control lane (with only siRNA) containing a single band represents the unbound siRNA. In contrast to the control, the immobile band will be observed upon successful polyplex formation. Additionally, this assay is indicative of how much of the siRNA is incorporated into the polyplexes. Optionally, further biophysical characterization of siRNA polyplexes, for example, to assign size and surface charge, is recommended and can be performed as described in Troiber et al. [17].

One of the most convenient models to screen RNAi-mediated gene silencing efficiency via RNAi in cell cultures is to use different reporter systems, for example, based on luciferase (Luc) or enhanced green fluorescent protein (eGFP). These reporter genes are indicative of functional gene silencing on the protein levels by measuring Luc or eGFP expression. In this, siRNA is designed to target the mRNA of the Luc or eGFP and upon efficient delivery of siRNA-containing polyplexes to the cytoplasm they induce RNAi-mediated gene silencing. We use siRNA targeting eGFP (siGFP) in the eGFP–Luc fusion construct and assign the gene silencing by measuring the luciferase activity by luminometric analysis. It is also worth mentioning that in this construct both transgenes could be targeted and used as an indicator for gene silencing activity. In RNAi measurements, it should be always remembered that control experiments with control siRNA sequences (siControl), which would not have any target in the cells, have their special role. This is due to the reason that siRNAs are associated with off-target activity (e.g. due to saturation of endogenous RNAi pathways or immunogenic effects on Toll-like receptors) and these controls give additional information on the potential toxicity of the polymers.

Many nonviral vectors are known to be toxic to the cells and therefore early screen for potential toxic side-effects are crucial. The most convenient assays for evaluation of general toxicity are different cell viability assays. We use CellTiter-Glo[®] assay to investigate whether the polyplexes are toxic to cells. In this assay, total amount of ATP are related to surviving cells and are exploited to produce light. The relative light units (RLU) are recorded as an indicator of cell viability to compare cells treated with siGFP polyplexes and controls.

3.1 Polyplex Formation

1. Thaw aliquots of polymer solution (5 mg/ml solution), siRNA (siGFP, siControl) solution (0.5 mg/ml), 20 mM HEPES buffered 5 % glucose pH 7.4 (HBG) on ice.
2. Prepare siRNA polyplexes at different *N/P* ratios using different polymers (*see Note 3*). The experiment is performed in triplicates. In the following example, we apply polymer **49** for the polyplex formation at *N/P* ratio 12:1.
 - (a) Combine 9 μ l of HBG and 1 μ l (500 ng) of siRNA solution in a 1.5 ml Eppendorf tube to obtain totally 10 μ l of siRNA-HBG dilution.
 - (b) Combine 9.26 μ l of HBG and 0.74 μ l (3.69 μ g) of **49** solution in another 1.5 ml Eppendorf tube to obtain 10 μ l of **49**-HBG dilution.
 - (c) Immediately add 10 μ l of **49**-HBG dilution into 10 μ l of siRNA-HBG dilution, then mix by rapidly pipetting up and down at least five times (*see Note 4*).
3. Allow the polyplex to form for 40 min exposure to air oxidation at room temperature in the closed Eppendorf tube (*see Note 5*).

3.2 siRNA Binding Assay

1. Prepare a standard 2 % agarose gel.
 - (a) Measure out 3 g of agarose powder.
 - (b) Pour 3 g of agarose powder into a microwavable flask along with 150 ml of 1 $\times$ TBE.
 - (c) Microwave for 1–3 min (until the agarose is completely dissolved and rolling boil appears).
 - (d) Add 15 μ l of GelRed[®] for siRNA staining in agarose gels. Gently shake the flask to ensure homogenous mixing.
 - (e) Let agarose solution cool down.
 - (f) Pour the agarose into a gel tray with the well comb in place (*see Note 6*).
 - (g) Let it at room temperature for 40 min until it has completely solidified.
 - (h) Once solidified, transfer the agarose gel to the electrophoresis chamber and remove the well comb.
 - (i) Add 1 $\times$ TBE into the electrophoresis chamber until the gel is covered.

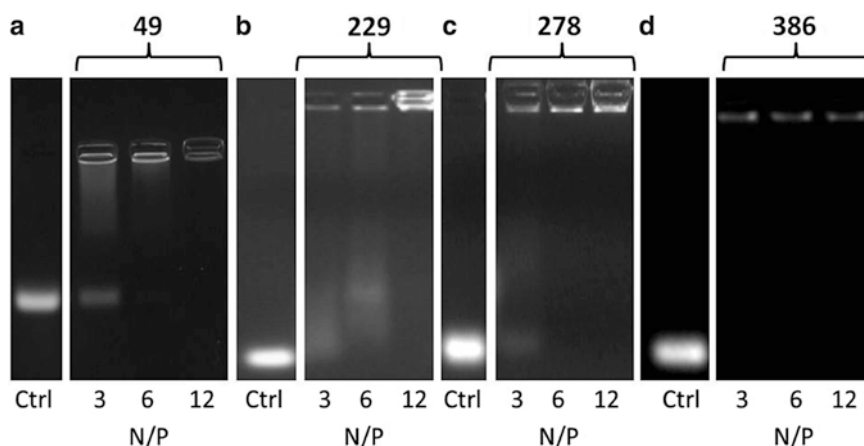


Fig. 3 siRNA binding assay at different N/P ratios. **(a, b)** Polymers **49** and **229** are able to bind and compact siRNA at N/P ratio of 12. **(c)** Polymer **278** displays high binding activity. **(d)** Most of the siRNA is retained in the gel pockets for the polymer **386** even at a low N/P ratio of 3. (Panels **b** and **d** reproduced from ref. [10] with permission from Elsevier; **c** reproduced from ref. [9] with permission from WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany)

2. Prepare siRNA-containing polyplexes at different N/P ratios (*see* Subheading 3.1). For control experiments, plain siRNA solution diluted in HBG is necessary.
3. Add 3 μ l of bromophenol blue as loading buffer into each sample.
4. Carefully load samples into wells of the gel.
5. Perform the electrophoresis at 80 V for 40 min.
6. Record the result with a standard UV transilluminator. Such assay can confirm successful complex formation and indicates N/P ratios where complete polyplex formation takes place, i.e. siRNA is fully incorporated into polyplexes (Fig. 3).

3.3 Cell Culture and Treatment

1. Different cell lines are used and cultured in the appropriate media (Table 2). For siRNA silencing assay, cells stably transfected with the eGFPLuc gene (Neuro2A/eGFPLuc cells, HepG2/eGFPLuc cells, or DU-145/eGFPLuc cells) are used. For cell viability assay, wild-type cells (Neuro2A cells, HepG2 cells, or DU-145 cells) are used. The cells are cultured in ventilated flasks inside incubators at 37 °C with 5 % CO₂ in a humidified atmosphere. Cell lines are allowed to grow until 80 % confluence and splitted when necessary.
2. 5×10^3 cells per well are seeded in 100 μ l of media using 96-well plates 24 h prior to the experiment.
3. Before the experiment, media is replaced with 80 μ l of fresh growth media.

4. Treatments are carried out by treating cells with 20 μ l of polyplex solution per well (*see* Subheading 3.1).
5. In parallel, it is imperative to run control experiments with untreated cells (as the gene silencing is measured against these untreated baseline values) and also treat cells with polyplexes containing siControl.
6. Gently shake the 96-well plates horizontally for few seconds for complete diffusion of polyplexes.
7. The cells are incubated at 37 °C. For siRNA silencing assay, the cells are incubated for 48 h. For cell viability assay, the cells are incubated for 24 and 48 h.

3.4 siRNA Silencing Assay

1. Cells are harvested and analyzed for gene silencing after 48 h incubations with siRNA-containing polyplexes (*see* Subheading 3.3).
2. Prepare cell lysis buffer.
 - (a) Thaw an aliquot of 5 $\times$ lysis reagent at ambient temperature, and equilibrate for at least 30 min.
 - (b) Add four volumes of water to one volume of 5 $\times$ lysis reagent to obtain 1 $\times$ lysis reagent.
3. Remove the transfection media.
4. Add 100 μ l of 1 $\times$ lysis reagent into each well to obtain cell lysate.
5. Measure the relative light units (RLU) of cell lysate by a plate-reading luminometer. We have used Centro LB 960 plate reader luminometer (Berthold, Germany), for the example below.
 - (a) Thaw an aliquot of 10 mM luciferin.
 - (b) Mix 500 μ l of 10 mM luciferin with 10 ml of luciferase assay reagent (LAR), and apply the injector into the mixture.
 - (c) The injector is programmed to add 100 μ l of luciferin–LAR solution per well, then the plate is read immediately.
 - (d) Transfer 35 μ l of cell lysate each well to white 96-well Microplate (Berthold, Germany).
 - (e) Measure the light produced for a period of 10 s.
6. The relative light units (RLU) are presented as percentage of the luciferase gene expression obtained with untreated control cells. Example of successful gene silencing is shown in Fig. 4.

3.5 Cell Viability Assay

1. Cells are harvested and analyzed for cytotoxicity after 24 and 48 h incubations with siRNA-containing polyplexes (*see* Subheading 3.3). For consistent results, equilibrate cells to room temperature before performing the assay.
2. Thaw CellTiter-Glo® Reagent.

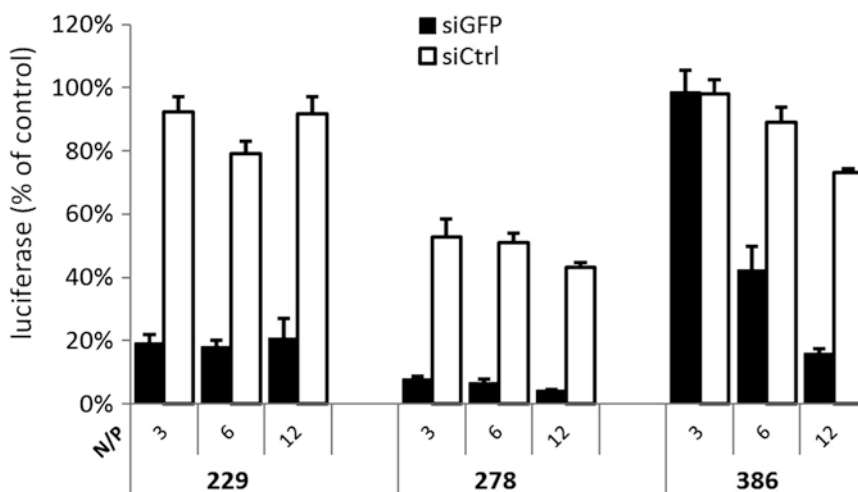


Fig. 4 Gene silencing ability of polyplexes in Neuro2A-eGFPLuc cells with siRNA targeted against eGFPLuc fusion protein (siGFP) or control siRNA (siControl) was tested (at different *N/P* ratios). Treatment with siGFP polyplexes (but not siControl polyplexes) shows effective gene silencing for polymers **229** and **278** over a broad range of *N/P* ratios. For polymer **386**, though it displays moderate knockdown activity at *N/P* ratio of 6, successful gene silencing is observed at *N/P* ratio of 12. Luciferase activity is determined for cells treated either with siGFP or with siControl and always compared (in relative %) with the luciferase activity (100 %) of untreated control cells (reproduced from ref. [10] with permission from Elsevier)

3. Add 100 μ l of CellTiter-Glo[®] Reagent to the media present in each well.
4. Place the plate on an orbital shaker for 10 min to induce cell lysis.
5. Transfer the mixture in each well to a white 96-well plate (Nunc, Germany) for measurement.
6. The luminescence is recorded with a plate-reading luminometer, e.g. Lumat LB 960 (Berthold, Germany). The relative light units (RLU) of treated cells are presented as percentage compared to the untreated control cells. Example of the cytotoxicity measurement and nontoxic nature of polymers **49**, **229**, and **278** is shown in Fig. 5.

4 Notes

1. To prevent freeze/thaw damage, we suggest preparing small volume of aliquots based on experimental design.
2. Light intensity is a measure of the rate of catalysis by luciferase and is therefore dependent upon temperature. The optimum temperature for luciferase activity is approximately room temperature (20–25 °C). It is important to fully equilibrate luciferase assay reagent to room temperature before measurements. The solution is stable for approximately 2 weeks at 4 °C.

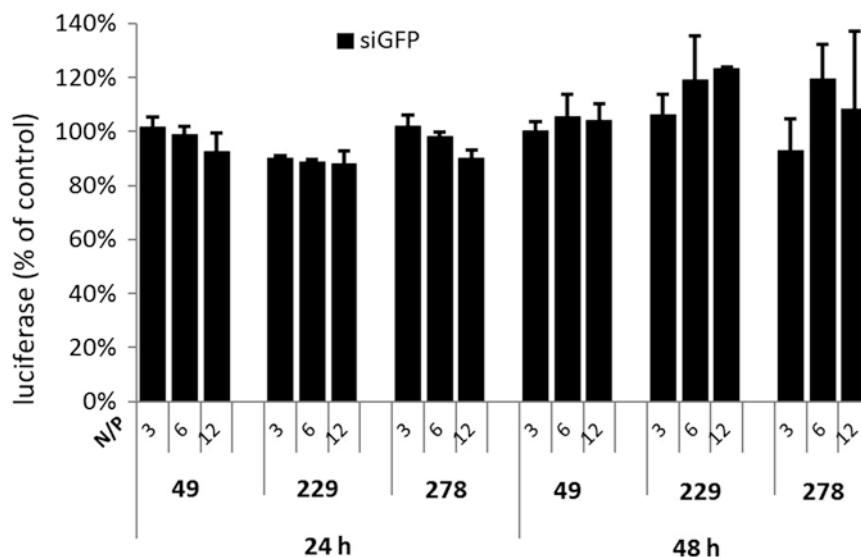


Fig. 5 Cell viability of polyplexes in Neuro2A cells with siRNA targeted against eGFP_{Luc} fusion protein (siGFP) was tested (at different *N/P* ratios) for 24 and 48 h. None of the siGFP polyplexes cause significant toxicity for the presented polymers **49**, **229**, and **278**. Even at the highest *N/P* ratio of 12, no or only very moderate reduction of the luciferase signal is observed. Luciferase activity is determined for cells treated with siGFP and always compared (in relative %) with the luciferase activity (100 %) of untreated control cells

Table 3
Number or pronatable amines in polymers used

Polymer	Pronatable amines	Molecular weight ^a
49	13	2,569.5
229	9	2,020
278	10	2,965.8
386	29	3,955.2
454	13	3,548.1

^aThe molecular weight of the polymer as HCl salt [9–11]

- The amount of polymer (n_{polymer} , nmole) for the fixed amount of nucleic acid is calculated via *N/P* (pronatable amines of the polymer/phosphates of the siRNA) ratio:

$$n_{\text{polymer}} (\text{nmole}) = \frac{m_{\text{siRNA}} (\text{ng})}{M_{\text{siRNA}}} \cdot P_{\text{siRNA}} \cdot \frac{N/P}{N_{\text{polymer}}}$$

m_{siRNA} : weight of siRNA (ng); M_{siRNA} : molecular weight of siRNA; P_{siRNA} : amount of phosphates of siRNA; (see Table 3); *N/P*: we usually test at *N/P* ratios 3:1–20:1.

- Avoid bubbles when pipetting.

5. Based on a pilot study monitoring disulfide formation [10], after 40 min of polyplex incubation time approximately 60 % of all free thiol groups were oxidized at N/P ratio of 6. The oxidation rate decreased with rising N/P ratios.
6. Pour slowly to avoid bubbles which will disrupt the gel. Any bubbles can be pushed away from the well comb or toward the edges of the gel with a pipette tip.

Acknowledgements

This work was supported by the German Research Foundation grant SFB1032 (project B4) and the excellence cluster Nanosystems Initiative Munich. D.J.L. was supported by the Bavarian Research Foundation PhD Scholarship.

References

1. Fire A et al (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–811
2. Elbashir SM et al (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494–498
3. Castanotto D, Rossi JJ (2009) The promises and pitfalls of RNA-interference-based therapeutics. *Nature* 457:426–433
4. Kanasty RL et al (2012) Action and reaction: the biological response to siRNA and its delivery vehicles. *Mol Ther* 20:513–524
5. Wagner E (2013) Biomaterials in RNAi therapeutics: quo vadis? *Biomater Sci* 1:804–809
6. Burnett JC, Rossi JJ (2012) RNA-based therapeutics: current progress and future prospects. *Chem Biol* 19:60–71
7. Dohmen C et al (2012) Nanosized multifunctional polyplexes for receptor-mediated siRNA delivery. *ACS Nano* 6:5198–5208
8. Singh S, Sharma A, Robertson GP (2012) Realizing the clinical potential of cancer nanotechnology by minimizing toxicologic and targeted delivery concerns. *Cancer Res* 72: 5663–5668
9. Schaffert D et al (2011) Solid-phase synthesis of sequence-defined T-, i-, and U-shape polymers for pDNA and siRNA delivery. *Angew Chem Int Ed Engl* 50:8986–8989
10. Frohlich T et al (2012) Structure-activity relationships of siRNA carriers based on sequence-defined oligo (ethane amino) amides. *J Control Release* 160:532–541
11. Troiber C et al (2013) Stabilizing effect of tyrosine trimers on pDNA and siRNA polyplexes. *Biomaterials* 34:1624–1633
12. Edinger D et al (2013) Gene silencing and antitumoral effects of Eg5 or Ran siRNA oligoaminoamide polyplexes. *Drug Deliv Transl Res* 4:84–95.
13. Morille M et al (2008) Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. *Biomaterials* 29: 3477–3496
14. Edinger D, Wagner E (2011) Bioresponsive polymers for the delivery of therapeutic nucleic acids. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 3:33–46
15. Scholz C, Wagner E (2012) Therapeutic plasmid DNA versus siRNA delivery: common and different tasks for synthetic carriers. *J Control Release* 161:554–565
16. Wagner E (2012) Polymers for siRNA delivery: inspired by viruses to be targeted, dynamic, and precise. *Acc Chem Res* 45:1005–1013
17. Troiber C et al (2013) Comparison of four different particle sizing methods for siRNA polyplex characterization. *Eur J Pharm Biopharm* 84:255–264

Regulatory Non-Coding RNAs

Methods and Protocols

Carmichael, G.G. (Ed.)

2015, X, 181 p. 39 illus., 10 illus. in color., Hardcover

ISBN: 978-1-4939-1368-8

A product of Humana Press