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Synthesis and Biological Evaluation of a Bioresponsive and Endosomolytic siRNA−Polymer Conjugate

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Abstract: Extracellular stability of electrostatically formed siRNA polyplexes is a significant concern in the delivery process. To overcome the risk of polyplex dissociation in the extracellular environment, siRNA was covalently incorporated into a pH- and redox-responsive polymer conjugate. The novel siRNA conjugate consists of polylysine (PLL) as RNA binding and protecting polycation, polyethylene glycol (PEG) as solubilizing and shielding polymer, the lytic peptide melittin masked by dimethylmaleic anhydride (DMMAn) removable at endosomal pH, and the siRNA attached at the 5′-end of the sense strand via a bioreducible disulfide bond. The purified siRNA conjugate was stable in the presence of the polyanion heparin at conditions where the analogous electrostatic siRNA polyplexes disassemble. Only the combination of heparin plus a reducing agent such as glutathione triggered the release of siRNA from the conjugate. High *in vitro* biocompatibility (absence of cytotoxicity or hemolytic activity at neutral pH) and efficient and sequence-specific gene silencing was found at ≥25 nM siRNA, comparable to the corresponding electrostatic polyplexes. *In vivo* toxicity studies of this formulation demonstrated that conjugates remain to be optimized for therapeutic application.

Keywords: Nucleic acid delivery; siRNA conjugate; bioresponsive; endosomolytic peptide; melittin

Introduction

siRNA mediated RNA interference (RNAi) has attracted considerable interest in research and therapeutic development. Andrew Fire and Craig Mello won in 2006 the Nobel Prize for their work on RNA interference in *Caenorhabditis elegans* which was published in the year 1998.[1] Tuschl and his colleagues published in 2001 that synthetic siRNAs can silence target genes in mammalian systems in a specific manner.[2] siRNAs function in the cytoplasm and are incorporated into an RNA-induced silencing complex (RISC). The antisense RNA strand, complementary to the target mRNA sequence, when incorporated in the RISC complex[3] guides mRNA cleavage in the middle of the sequence.[4] Although first siRNA therapeutics entered clinical trials, there is still a great need for powerful delivery strategies to exploit the full therapeutic potential of siRNA. Especially in vivo

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(1) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis elegans. *Nature (London)* **1998**, *391*, 806–811.

(2) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. Duplexes of 21-Nucleotide RNAs Mediate RNA Interference in Cultured Mammalian Cells. *Nature (London)* **2001**, *411*, 494–498.

(3) Wang, Y.; Sheng, G.; Juranek, S.; Tuschl, T.; Patel, D. J. Structure of the Guide-Strand-Containing Argonaute Silencing Complex. *Nature (London)* **2008**, *456*, 209–213.

(4) Bumcrot, D.; Manoharan, M.; Koteliansky, V.; Sah, D. W. RNAi Therapeutics: a Potential New Class of Pharmaceutical Drugs. *Nat. Chem. Biol.* **2006**, *2*, 711–719.

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delivery remains a major challenge and requires further formulation development efforts.

siRNA formulations include electrostatic complexes of siRNA with cationic lipids (lipoplexes) or polycations (polyplexes), see reviews,[5−7] lipopolyplexes,[8−10] liposomes encapsulating siRNA[11] and siRNA conjugates.[12−14] The delivery systems can be equipped with functional domains, e.g. endosomolytic domains or targeting ligands.[10,15−18] To reach the site of action within the target cell, the nucleic acid has to stay associated with its carrier during the complete extracellular delivery process. Conventional polyplex formulations are held together electrostatically. Other charged molecules can disrupt such complexes before they reach the target cell. Burke et al.[19] demonstrated that both the serum and the extracellular matrix can lead to vector disassembly. They observed significant vector unpackaging of systemically injected fluorescence-labeled polyethylenimine (PEI)/plasmid DNA (pDNA) polyplexes in the liver. Additionally, exposing polyplexes to serum or proteoglycans before *in vitro* transfection caused decreased cellular uptake of the nucleic acid.[19] The work of Buyens et al.[20] addressed the same weak point of extracellular dissociation for siRNA complexes. Using fluorescence correlation spectroscopy to study the dissociation they demonstrated that siRNA lipoplexes undergo fast disassembly in full human serum. The authors mention in their paper similar disassembly for PEI/siRNA polyplexes.[20] It can be assumed that vector unpacking is even more likely a problem in the case of siRNA in comparison with pDNA, as the far larger number of negative charges of pDNA stabilizes the interelectrolyte complex.[21,22]

We have recently described pH-responsive endosomolytic conjugates based on the polycations PEI or polylysine (PLL) modified with polyethylene glycol (PEG) and dimethylmaleic anhydride (DMMAn) masked melittin (DMMAn-Mel). The conjugates formed polyplexes with siRNA and pDNA and achieved excellent gene knockdown efficiencies.[23] However, as we show in the current work, unpacking of these polyplexes can be observed by treatment with heparin. To overcome this drawback we now covalently attached the siRNA to the PLL backbone. This paper describes the synthesis and purification of a pH- and redox-sensitive

(5) Aigner, A. Gene Silencing Through RNA Interference (RNAi) in Vivo: Strategies Based on the Direct Application of siRNAs. *J. Biotechnol.* **2006**, *124*, 12–25.
(6) Meyer, M.; Wagner, E. Recent Developments in the Application of Plasmid DNA-Based Vectors and Small Interfering RNA Therapeutics for Cancer. *Hum. Gene Ther.* **2006**, *17*, 1062–1076.
(7) Gary, D. J.; Puri, N.; Won, Y. Y. Polymer-Based siRNA Delivery: Perspectives on the Fundamental and Phenomenological Distinctions From Polymer-Based DNA Delivery. *J. Controlled Release* **2007**, *121*, 64–73.
(8) Gao, K.; Huang, L. Nonviral Methods for siRNA Delivery. *Mol. Pharmaceutics*, DOI: 10.1021/mp800134q, ASAP Dec 30, 2008.
(9) Li, S. D.; Chen, Y. C.; Hackett, M. J.; Huang, L. Tumor-Targeted Delivery of siRNA by Self-Assembled Nanoparticles. *Mol. Ther.* **2008**, *16*, 163–169.
(10) Chono, S.; Li, S. D.; Conwell, C. C.; Huang, L. An Efficient and Low Immunostimulatory Nanoparticle Formulation for Systemic siRNA Delivery to the Tumor. *J. Controlled Release* **2008**, *131*, 64–69.
(11) Zimmermann, T. S.; Lee, A. C.; Akinc, A.; Bramlage, B.; Bumcrot, D.; Fedoruk, M. N.; Harborth, J.; Heyes, J. A.; Jeffs, L. B.; John, M.; Judge, A. D.; Lam, K.; McClintock, K.; Nechev, L. V.; Palmer, L. R.; Racie, T.; Rohl, I.; Seiffert, S.; Shanmugam, S.; Sood, V.; Soutschek, J.; Toudjarska, I.; Wheat, A. J.; Yaworski, E.; Zedalis, W.; Koteliansky, V.; Manoharan, M.; Vornlocher, H. P.; MacLachlan, I. RNAi-Mediated Gene Silencing in Non-Human Primates. *Nature (London)* **2006**, *441*, 111–114.
(12) Oishi, M.; Nagasaki, Y.; Itaka, K.; Nishiyama, N.; Kataoka, K. Lactosylated Poly(Ethylene Glycol)-siRNA Conjugate Through Acid-Labile Beta-Thiopropionate Linkage to Construct pH-Sensitive Polyion Complex Micelles Achieving Enhanced Gene Silencing in Hepatoma Cells. *J. Am. Chem. Soc.* **2005**, *127*, 1624–1625.
(13) Moschos, S. A.; Jones, S. W.; Perry, M. M.; Williams, A. E.; Erjefalt, J. S.; Turner, J. J.; Barnes, P. J.; Sproat, B. S.; Gait, M. J.; Lindsay, M. A. Lung Delivery Studies Using siRNA Conjugated to TAT(48−60) and Penetratin Reveal Peptide Induced Reduction in Gene Expression and Induction of Innate Immunity. *Bioconjugate Chem.* **2007**, *18*, 1450–1459.
(14) Rozema, D. B.; Lewis, D. L.; Wakefield, D. H.; Wong, S. C.; Klein, J. J.; Roesch, P. L.; Bertin, S. L.; Reppen, T. W.; Chu, Q.; Blokhin, A. V.; Hagstrom, J. E.; Wolff, J. A. Dynamic PolyConjugates for Targeted in Vivo Delivery of siRNA to Hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12982–12987.
(15) Song, E.; Zhu, P.; Lee, S. K.; Chowdhury, D.; Kussman, S.; Dykxhoorn, D. M.; Feng, Y.; Palliser, D.; Weiner, D. B.; Shankar, P.; Marasco, W. A.; Lieberman, J. Antibody Mediated in Vivo Delivery of Small Interfering RNAs Via Cell-Surface Receptors. *Nat. Biotechnol.* **2005**, *23*, 709–717.
(16) Bartlett, D. W.; Su, H.; Hildebrandt, I. J.; Weber, W. A.; Davis, M. E. Impact of Tumor-Specific Targeting on the Biodistribution and Efficacy of siRNA Nanoparticles Measured by Multimodality in vivo Imaging. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 15549–15554.
(17) Philipp, A.; Meyer, M.; Wagner, E. Extracellular Targeting of Synthetic Therapeutic Nucleic Acid Formulations. *Curr. Gene Ther.* **2008**, *8*, 324–334.
(18) Tietze, N.; Pelisek, J.; Philipp, A.; Roedl, W.; Merdan, T.; Tarcha, P.; Ogris, M.; Wagner, E. Induction of Apoptosis in Murine Neuroblastoma by Systemic Delivery of Transferrin-Shielded siRNA Polyplexes for Downregulation of Ran. *Oligonucleotides* **2008**, *18*, 161–174.
(19) Burke, R. S.; Pun, S. H. Extracellular Barriers to in vivo PEI and PEGylated PEI Polyplex-Mediated Gene Delivery to the Liver. *Bioconjugate Chem.* **2008**, *19*, 693–704.
(20) Buyens, K.; Lucas, B.; Raemdonck, K.; Braeckmans, K.; Vercammen, J.; Hendrix, J.; Engelborghs, Y.; De Smedt, S. C.; Sanders, N. N. A Fast and Sensitive Method for Measuring the Integrity of siRNA-Carrier Complexes in Full Human Serum. *J. Controlled Release* **2008**, *126*, 67–76.
(21) Bolcato-Bellemin, A. L.; Bonnet, M. E.; Creusat, G.; Erbacher, P.; Behr, J. P. Sticky Overhangs Enhance siRNA-Mediated Gene Silencing. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16050–16055.
(22) Zintchenko, A.; Philipp, A.; Dehshahri, A.; Wagner, E. Simple Modifications of Branched PEI Lead to Highly Efficient siRNA Carriers With Low Toxicity. *Bioconjugate Chem.* **2008**, *19*, 1448–1455.
(23) Meyer, M.; Philipp, A.; Oskuee, R.; Schmidt, C.; Wagner, E. Breathing Life into Polycations: Functionalization With pH-Responsive Endosomolytic Peptides and Polyethylene Glycol Enables siRNA Delivery. *J. Am. Chem. Soc.* **2008**, *130*, 3272–3273.

siRNA conjugate with an endosomolytic peptide and PEG-modified polylysine. The covalent conjugate was compared with the analogous electrostatical polyplex in its stability against heparin, disassembly under reducing conditions, and gene knockdown activity in cell culture.

Materials and Methods

Reagents. Poly-L-lysine-hydrobromide (PLL, MW = 32000, degree of polymerization = 153), succinimidyl 3-(2-pyridyldithio) propionate (SPDP), 2,3-dimethylmaleicanhydride (DMMAn), 1,4-dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), ethidium bromide (EtBr), methylthiazolyldiphenyl-tetrazolium bromide (MTT), succinimidyl propionate monomethoxy polyethylene glycol (SPA-mPEG, molecular weight 5000), RNase-free water, absolute ethanol, deuterium oxide (D_2O) and dimethyl sulfoxide puriss (DMSO) were obtained from Sigma-Aldrich (Munich, Germany). Water was used as purified, deionized water.

Cysteine-modified melittin (Mel) was obtained from IRIS Biotech GmbH (Marktredwitz, Germany). Mel had the sequence CIGA VLKV LTTG LPAL ISWI KRKR QQ (all-D configuration). All-D stereochemistry was used because it is nonimmunogenic while being as lytic as the natural peptide.

Ready to use siRNA duplexes were purchased from Dharmacon (Lafayette, CO), namely, luciferase-siRNA [GL3 luciferase duplex: 5′-CUUACGCUGAGUACUUCGAdTdT-3′ (sense)]; control-siRNA [nontargeting control duplex: 5′-AUGUAUUGGCCUGUAUUAGUU-3′ (sense)]; thiol-modified GL3 siRNA had the sequence 5′-thiol-CUUACGCUGAGUACUUCGAdTdT-3′ (sense); thiol-modified control-siRNA had the sequence 5′-thiol-AUGUAUUGGCCUGUAUUAGUU-3′ (sense).

Cell culture media, antibiotics, and fetal calf serum were purchased from Invitrogen (Karlsruhe, Germany). Luciferase cell culture lysis buffer and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany).

Conjugate Synthesis. *Synthesis of PEG-Modified PLL.* PLL (1.25 μmol; DP = 153) in 2 mL of buffer (0.5 M NaCl, 20 mM HEPES, pH 7.4) was mixed with mPEG-SPA (1.6 μmol, 8 mg) dissolved in 400 μL of DMSO. After 2 h at RT the reaction mixture containing modified PLL (PLL-PEG) was loaded on a cation-exchange column (MacroPrep High S; HR 10/10, BioRad, München, Germany) and fractionated with a salt gradient from 0.6 to 3.0 M NaCl in 20 mM HEPES pH 7.4. The flow rate was 0.5 mL/min. The fractions containing PLL-PEG were pooled, dialyzed against water (MWCO of 6000−8000) and lyophilized. The degree of modification of PLL with PEG was determined by proton NMR (PLL/PEG = 1/1). The PLL content was measured by TNBS assay.

Synthesis of 3-(2-Pyridyldithio)propionate-Modified PLL-PEG. PLL-PEG (0.313 μmol, containing 6.45 mg of PLL as free base) in 2 mL of buffer (20 mM HEPES, pH 7.4) was mixed with SPDP (3.8 μmol, 1.19 mg) dissolved in 200 μL of DMSO. After 2 h at RT PLL-PEG with pyridyldithiopropionate-linkers (PEG-PLL-PDP) was purified by gel filtration using an Äkta Basic HPLC System (Amersham Biosciences, Freiburg, Germany) equipped with a Sephadex G-25 superfine HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 0.5 M NaCl, 20 mM HEPES, pH 7.4; the flow rate was 1 mL/min. The fractions containing PEG-PLL-PDP were pooled, and aliquots were snap frozen in liquid nitrogen and stored at −80 °C. PLL content of the fractions was measured by TNBS assay. The degree of modification with the dithiopyridine linker was determined spectrophotometrically at 343 nm by release of pyridine-2-thione: 10 μL of the sample was added to 140 μL of water, and blank absorbance at 343 nm was measured. Subsequently 5 μL of the reducing agent DTT (0.5 μmol, 77 μg; diluted in water) was added and release of pyridine-2-thion at 343 nm (molar absorptivity = 8080 M^{-1} cm^{-1}) was measured. Two PEG-PLL-PDP batches were prepared with molar ratios of 1/1/8 and 1/1/11.

Synthesis of DMMAn-Mel-Modified PLL-PEG. Mel peptide (1.38 μmol, 4 mg) was dissolved in 400 μL of 100 mM HEPES and 125 mM NaOH and mixed with 1000 μL of ethanol containing 15.8 μmol (2 mg) of DMMAn under argon for 0.5 h, following purification and concentration to 200 μL via ultrafiltration (Vivascience, Vivaspin 2, MWCO 2000 HY). 1.38 μmol of the acylated melittin in 200 μL was mixed under argon with 1.06 mL of PEG-PLL-PDP (116 nmol of PLL, 2.39 mg of PLL, molar ratio of PLL/PEG/PDP of approximately 1/1/8) diluted in 2 M guanidine hydrochloride, 0.5 M NaCl, 20 mM HEPES, pH 8. After 2 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction. The degree of modification was determined at 343 nm by release of pyridine-2-thione from residual PDP linkers after reduction with DTT. PEG-PLL-DMMAn-Mel conjugates were purified on the Äkta Basic HPLC System equipped with a Superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 0.5 M NaCl, 20 mM HEPES, pH 8. The flow rate was 0.5 mL/min. The void fractions containing PEG-PLL-DMMAn-Mel (molar ratio of PEG/PLL/DMMAn-Mel of approximately 1/1/8) were pooled, and aliquots were snap frozen in liquid nitrogen and stored at −80 °C. The PLL content of the conjugate was determined by TNBS assay.

Deprotection of Thiol-Modified siRNA. Thiol-modified siRNA was shipped with the thiol group in a protected/oxidized form to prevent the formation of dimers. Prior to use, the thiol groups of the thiol-modified siRNA were reduced. 188 nmol of lyophilized siRNA was incubated for 1 h at RT with 400 μL of 0.05 M TCEP-solution under shaking. 150 μL of 9.5 M ammonium acetate and 1.5 mL of ice cold ethanol were added, and the solution was incubated for 20 min at −80 °C. Subsequently the sample containing precipitated siRNA was centrifuged at 13400 rpm for 20 min. After the supernatant was poured off, the sample was air-dried for 15 min and the pellet reconstituted in 200 μL of RNase-free water. For complete removal of TCEP the

precipitation procedure was repeated. siRNA quantification was carried out by obtaining an absorbance at 260 nm. The free thiol groups were quantified by Ellman's assay.

Synthesis of PEG-PLL-DMMAn-Mel-siRNA. An aqueous solution of 672 μL of PEG-PLL-PDP (0.076 μmol of PLL, 0.84 μmol of PDP, molar ratios of PEG/PLL/PDP = 1/1/11) was mixed with 0.10 μmol of thiol-siRNA (1.35 mg) and incubated for 1 h at RT under argon. Before addition of siRNA, the solution was adjusted to 1.5 M NaCl. After 1 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction and degree of modification with siRNA. Subsequent DMMAn-Mel coupling was carried out analogously to DMMAn-Mel-modified PLL-PEG except high salt concentration (1.5 M NaCl) was used instead of guanidine hydrochloride. PEG-PLL-DMMAn-Mel-siRNA conjugates were purified on the Äkta Basic HPLC System equipped with a Superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 1 M NaCl, 20 mM HEPES, pH 8. The flow rate was 0.5 mL/min. The void fractions containing the conjugate were pooled, and aliquots were snap frozen in liquid nitrogen and stored at −80 °C. The PLL content of the conjugate was determined by TNBS assay. The yield of the conjugate (molar ratio of PEG/PLL/DMMAn-Mel/siRNA of approximately 1/1/7.5/1.5) was about 60% based on analysis of PLL, which is comparable to yields obtained in the synthesis of PEG-PLL-DMMAn-Mel.

For subsequent experiments the synthesis was further optimized. In short, buffers and solvents were adjusted to eliminate the risk of DMMAn-Mel precipitation and oxidation during the synthesis. *DMMAn-Mel Preparation:* Mel peptide (0.69 μmol, 2 mg) was dissolved in 200 μL of 250 mM HEPPS pH 8.2, 30% (v/v) acetonitrile, and mixed with 10 μL of acetonitrile containing 7.93 μmol (1 mg) of DMMAn. After incubation for 0.5 h under argon excess DMMAn was quenched by adding 35 mg of lysine in 35 μL of 250 mM HEPPS, pH 8.2. *siRNA Conjugation:* An aqueous solution of 509 μL of PEG-PLL-PDP (0.051 μmol of PLL, 0.407 μmol of PDP, molar ratios of PEG/PLL/PDP = 1/1/8) was mixed with 0.061 μmol of thiol-siRNA (0.82 mg) and incubated for 1 h at RT under argon. Before addition of siRNA, the solution was adjusted to 1.5 M NaCl. After 1 h at RT, released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction and degree of modification with siRNA. Acetonitrile was added to the siRNA solution to a content of 30% (v/v). *DMMAn-Mel Coupling:* The PEG-PLL-PDP-siRNA and DMMAn-Mel solutions were mixed and incubated for 2 h at RT under argon for coupling. PEG-PLL-DMMAn-Mel-siRNA conjugates were purified on the Äkta Basic HPLC System equipped with a Superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 1 M NaCl, 20 mM HEPES, pH 8. The flow rate was 1.0 mL/min. The void fractions containing the conjugate were pooled and directly dialyzed against HBG (20 mM HEPES, 5% glucose w/v, pH 8) using SPECTRAPOR dialysis tube, cutoff 6−8 kDa). The yield of the conjugate (molar ratio of PEG/PLL/DMMAn-Mel/siRNA of approximately 1/1/6/1) was about 65% based on analysis of PLL.

siRNA conjugates generated by the two procedures had indistinguishable properties regarding lytic activity, transfection efficiency, and *in vitro* and *in vivo* toxicity (data not shown).

Quantitative Analysis of Poly-L-lysine (TNBS Assay). The concentration of PLL was measured by TNBS assay. Standard PLL solutions and test solutions containing polycation were serially diluted in 0.1 M sodium tetraborate to a final volume of 100 μL using a 96-well plate, resulting in PLL hydrobromide concentrations of 10 to 60 μg/mL. To each well was added 2.5 μL of TNBS (75 nmol, 22 μg; diluted in water). TNBS reacts with primary amino groups to form colored trinitrophenylated derivatives. After 5−20 min incubation time at RT (depending on the strength of the developed color) the absorption was measured at 405 nm using a microplate reader (Spectraflour Plus, Tecan Austria GmbH).

Ellman's Assay. This assay is a colorimetric reaction between Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), DTNB) and the free thiol groups. 0.4 mg of DTNB dissolved in 1 mL of the corresponding Ellman's buffer (0.2 M Na_2HPO_4 with 1 mM EDTA at pH 8.0) was used as stock solution. For vis absorption measurement Ellman's stock diluted 1:10 in Ellman's buffer was taken as blank. Standard cystein solution and samples were serially diluted in Ellman's buffer and 10% (v/v) of the stock solution. After 20 min at 37 °C the solutions were measured at 412 nm and concentration of the thiol group was calculated via the standard curve.

Polyplex Formation. Noncovalent polyplex formulations for siRNA delivery were prepared as follows: the appropriate amounts of siRNA and PEG-PLL-DMMAn-Mel conjugate (as indicated in the corresponding experiments) were diluted in separate tubes in HBG. Then, the polycation solution was added to the siRNA, mixed by pipetting up and down and incubated for 20−30 min at RT to form the siRNA polyplexes that were used for transfection experiments. In case of the conjugate containing covalently attached siRNA, the conjugate was diluted with HBG to the adequate transfection concentration.

Measurement of Particle Size via Dynamic Light Scattering (DLS). Particle size of siRNA formulations was measured by laser-light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). The siRNA conjugate was measured undiluted or diluted with HBG or NaCl solution before measurement.

Agarose Gel Electrophoresis. A 2.5% agarose gel was prepared by dissolving 1 g of agarose (Sigma-Aldrich, Taufkirchen, Germany) in 40 g of TBE buffer (trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g and 1000 mL of water) and boiling everything up to 100 °C. After cooling down to about 50 °C and addition of ethidium bromide, the agarose gel was casted in the electrophoresis unit. Sample preparations containing 500 ng of siRNA and loading buffer (6 mL of glycerine, 1.2 mL of 0.5 M EDTA, 2.8 mL of H_2O, 0.02 g of xylenecyanole) were placed into

the sample pockets. For reduction of disulfides samples were treated with TCEP (50 mM) or glutathione (1.25−5 mM). To inhibit electrostatic interactions between the nucleic acid and the polycation, samples were treated with heparin (2 IU/20 μL). Electrophoresis was performed at 80 V for 40 min. Alternatively a 2.5% agarose gel with 120 g of TBE buffer was prepared and settings were adapted.

Erythrocyte Leakage Assay. Murine erythrocytes were isolated from fresh, citrate buffered blood and washed with phosphate-buffered saline (PBS) several times. The erythrocyte pellet was diluted 1000-fold in HBG or HBG containing 3% FCS to obtain a concentration of approximately 10^7 erythrocytes per mL. PEG-PLL-DMMAn-Mel-siRNA conjugates were serially diluted in 75 μL of HBG or HBG containing 3% FCS using a V-bottom 96-well plate (NUNC, Denmark). For 100% lysis, control wells contained buffer with 1% Triton X-100. A volume of 75 μL of erythrocyte suspension was added to each well, and the plates were incubated at 37 °C under constant shaking for 10 min in the case of HBG or 20 min in the case of HBG containing 3% FCS, respectively. After centrifugation 80 μL of the supernatant was analyzed for hemoglobin release at 405 nm using a microplate plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria).

Cell Culture. Cell culture media, antibiotics and fetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere. Neuro2A-eGFPLuc cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1 g/L glucose) containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine.

Luciferase Gene Silencing. All experiments were performed in stably transfected Neuro2A-eGFPLuc cells. Cells were seeded in 96-well plates (TPP, Trasadingen, Switzerland) 24 h prior to transfection using 5000 cells per well (resulting in approximately 9000 cells per well at the day of transfection). Then transfection complexes containing siRNA were added to the cells in 100 μL of culture medium containing 10% serum, 100 U/mL penicillin and 100 μg/mL streptomycin. At 48 h after initial transfection medium was removed and cells were lysed in 50 μL of Promega cell lysis solution to measure the gene expression. Luciferase activity was measured using a Lumat LB9507 instrument (Berthold, Bad Wildbad, Germany). Luciferase light units were recorded from a 20 μL aliquot of the cell lysate with 10 s integration time after automatic injection of freshly prepared luciferin using the luciferase assay system (Promega, Mannheim, Germany). Transfection efficiency was evaluated as relative light units (RLU) per number of seeded cells. Transfections were also performed with a nonspecific control siRNA to distinguish between specific gene silencing and unspecific knockdown of protein expression due to carrier toxicity. Qualitative judgment on the toxicity of the conjugates was made by diminution in luciferase expression upon delivery of the nonspecific control siRNA compared to the luciferase expression from the same number of cells that were not exposed to the carrier.

Cell Viability Assay. Metabolic activity of cells was determined using a methylthiazole tetrazolium (MTT)/thiazolyl blue assay as follows: 10 μL of a 5 mg/mL solution of MTT in sterile PBS buffer was added to each well of the 96-well plate. After incubation for 2 h at 37 °C in 5% CO_2, the medium was removed and cells were frozen for 2 h (−80 °C). 100 μL of DMSO was added, and samples were further incubated at 37 °C for 30 min under constant shaking. Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a microplate reader (Spectrafluor Plus), and cell viability was expressed as a percent relative to buffer-treated control cells.

***In Vivo* Experiments.** A/J mice were purchased from Harlan Winkelmann (Borchen, Germany) and housed in individually vented cages; food and water were provided ad libitum. For toxicity studies, tumor free mice (7 weeks old) were injected intravenously via tail vein with conjugates containing 25 or 12.5 μg of siRNA in 250 μL of HBG solution per 20 g of body weight. For intratumoral application studies 6-week-old mice were inoculated subcutaneously in the flank with 1×10^6 Neuro2A-GL3Luc cells in 100 μL of PBS. siRNA experiments started when tumors reached diameter of 8 to 10 mm in size. Mice were anesthetized with 2.5% isofluorane (Abbott, Wiesbaden, Germany) in oxygen. Intratumoral application of luciferase siRNA conjugates was performed by slowly injecting conjugates containing 25 μg of siRNA in 100 μL of HBG solution into the tumor tissue using a 30 g insulin syringe (Becton Dickinson, Heidelberg, Germany). Animal experiments were performed according to guidelines of the German law of protection of animal life and were approved by the local animal experiments ethical committee.

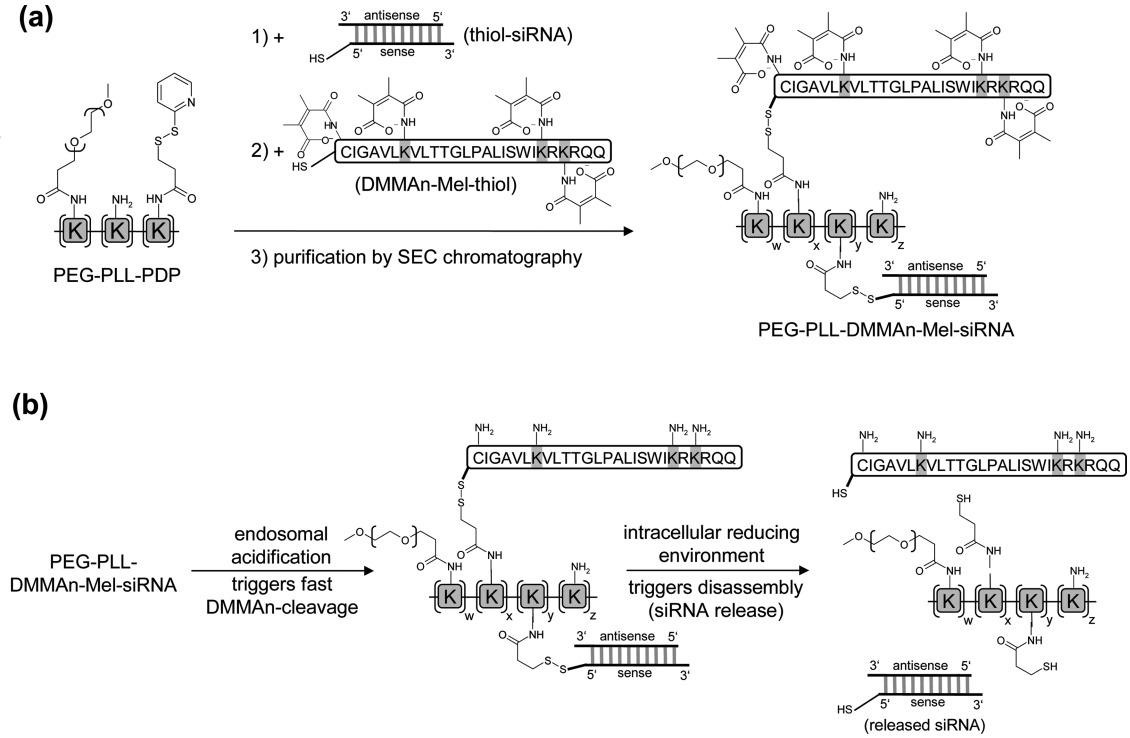
Results

Design and Synthesis of the PEG-PLL-DMMAn-Mel-siRNA Conjugate. Nonviral nucleic acid vectors are commonly generated by the complexation of negatively charged nucleic acids with cationic polymers or cationic lipids. In our previous work the polylysine conjugate PEG-PLL-DMMAn-Mel was synthesized which turned out to be a promising siRNA carrier.[23] Poly-L-lysine was applied as siRNA binding polycation, PEG as shielding and solubilizing molecule; the DMMAn groups are cleaved from the conjugated melittin peptides upon endolysosomal acidification, expose the lytic activity of melittin,[24] and should enable intracellular release out of the endosomes. However, interaction of polyplexes with polyanions, e.g. negative heparan sulfates of the extracellular matrix or charged plasma proteins like soluble glycosaminoglycans, may lead to vector unpackaging before the nucleic acid carrier reaches the target cell.[19]

To ensure extracellular stability, we synthesized a novel conjugate containing siRNA covalently attached to PEG-

(24) Meyer, M.; Zintchenko, A.; Ogris, M.; Wagner, E. A Dimethylmaleic Acid-Melittin-Polylysine Conjugate With Reduced Toxicity, pH-Triggered Endosomolytic Activity and Enhanced Gene Transfer Potential. *J. Gene Med.* **2007**, *9*, 797–805.

Scheme 1[a]



[a] (a) Synthesis of PEG-PLL-DMMAn-Mel-siRNA. (b) pH triggered cleavage of DMMAn masking groups and release of siRNA upon disulfide cleavage in reducing environment. [K] refers to the L-lysine monomers of the pLL backbone. The one-letter-code sequence of melittin (Mel) refers to the all-D-amino acid configuration. Substitutions (w, x, y, z) are random (not block) in sequence. Approximate ratio w/x/y/z for the described conjugates is 1:6-8:1−1.5:140−145.

PLL-DMMAn-Mel (see Scheme 1a). As covalent linkage a bioreversible disulfide bond between the 5′ end of the sense strand of thiolated siRNA and mercaptopropionic acid-modified PLL was chosen. After reaching the cytosol the intracellular reductive environment could cleave the disulfide bond and thus enable siRNA release and subsequent RISC activation (see Scheme 1b).

Synthesis of PEG-PLL-DMMAn-Mel-siRNA (Scheme 1a) was carried out similarly to the PEG-PLL-DMMAn-Mel synthesis.[23] Poly-L-lysine (PLL, MW 32 kDa, hydrobromide salt) was first modified with PEG (5 kDa)-SPA (1 molar equiv incorporated) and SPDP (8−11 molar equiv incorporated). Thiol-modified luciferase siRNA (sense strand modified at the 5′ end) was mixed with the pyridyldithiopropionate (PDP)-modified PEGylated PLL. After reaction of thiol-siRNA with PDP-linkers in the first step, the thiol-containing DMMAn-Mel was added to the mixture in the second reaction step. To avoid electrostatic aggregation of anionic siRNA and DMMAn-Mel with the cationic PEG-PLL-PDP during synthesis, a high salt concentration (1.5 M NaCl) was applied.

To clarify whether unbound siRNA (MW 13400) can be separated from the conjugate by size exclusion chromatography, first a mock synthesis without covalent siRNA linkage was carried out. Unmodified siRNA (without thiol group) was mixed with PEG-PLL-PDP and DMMAn-Mel as described above. The reaction mixture containing PEG-PLL-DMMAn-Mel-PDP, free siRNA, DMMAn-Mel and low molecular weight impurities was loaded onto a Superdex 75 column (Figure 1a). Analysis of separated fractions by agarose gel electrophoresis (Figure 1b) clearly shows that separation of the mixture was possible, with the PEG-PLL-DMMAn-Mel eluting as peak I and free siRNA as peak II.

As expected, mixing of siRNA and PEG-PLL-DMMAn-Mel led to complex formation which prevented siRNA movement into the gel (Figure 1b, lane 2). However electrostatic interactions could be eliminated by heparin (Figure 1b, lane 3). siRNA was only present in peak II (Figure 1b, lanes 6 and 7) and hence well separated from the PEG-PLL-DMMAn-Mel conjugate present in peak I (Figure 1b, lanes 4 and 5) by SEC.

After establishing the reaction and purification protocol, synthesis was conducted analogously with the thiol-modified siRNA. First thiol-modified GL3 luciferase siRNA was mixed with PEG-PLL-PDP. After 1 h released pyridine-2-thione was measured at 343 nm to determine the degree of modification with siRNA (molar ratio: approximately 1.5 siRNA per PLL). Subsequently DMMAn-Mel was coupled to the remaining PDP groups and the siRNA conjugate was loaded onto a Superdex 75 size exclusion column for purification. Figure 2a shows the result of the SEC purification.

A much smaller but significant amount of free siRNA (peak II) was found in comparison with the mock synthesis

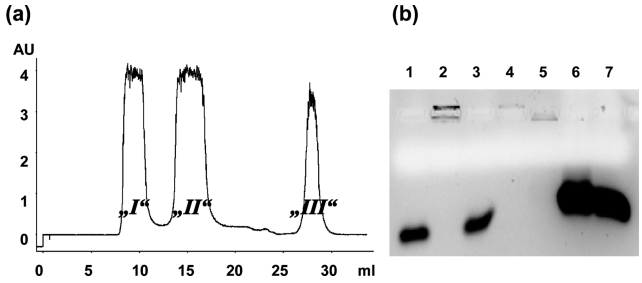


Figure 1. Chromatogram of PEG-PLL-DMMAn-Mel/siRNA mixture and gel electrophoresis analysis. (a) The mixture containing the PEG-PLL-DMMAn-Mel conjugate and unmodified siRNA was loaded onto a Superdex 75 HR 10/30 column. The conjugate was eluted with 1 M NaCl, 20 mM Hepes, pH 8.2; flow rate, 0.5 mL/min; *x*-axis, elution volume; *y*-axis, absorption at 280 nm. Peak I, PEG-PLL-DMMAn-Mel conjugate; peak II, unbound siRNA; peak III, pyridine-2-thione (released from linker during coupling reaction). (b) Agarose gel electrophoresis of peaks I and II. Lane 1, 0.5 μg siRNA (control, in HBG); lane 2, PEG-PLL-DMMAn-Mel/siRNA polyplex (w/w 2/1, prepared in HBG); lane 3, like 2 + heparin; lane 4, 20 μL of peak I; lane 5, 20 μL of peak I + heparin; lane 6, 20 μL of peak II; lane 7, 20 μL of peak II + heparin.

(described in Figure 1a), indicating conjugation of most (but not all) thiol-modified siRNA to PEG-PLL-PDP. The fractions containing the conjugate (peak I) were pooled and snap-frozen in liquid nitrogen. The purified PEG-PLL-DMMAn-Mel-siRNA conjugate had a molar ratio of PLL/PEG/DMMAn-Mel/siRNA = 1/1/7.5/1.5. This presents a PLL/siRNA w/w ratio of 1.6/1, close to the optimum ratio found for the corresponding siRNA polyplexes.[23] Analogously a conjugate containing nonsilencing control siRNA was synthesized.

Agarose Gel Electrophoresis of Conjugate. Covalent attachment of siRNA or rather absence of free siRNA in the conjugate was confirmed by agarose gel electrophoresis (Figure 2b). From untreated conjugate (Figure 2b, lane 4) no siRNA is released during agarose gel electrophoresis. Neither TCEP (reducing agent) nor heparin (polyanion) pretreatment alone could induce a release of siRNA (Figure 2b, lane 5 and 6, respectively), indicating that siRNA is associated with PLL both covalently and electrostatically. The observed smear in lane 6 is presumably due to heparin coating of the polycationic conjugate and subsequent movement of the overall negatively charged aggregate into the gel. Only if reducing conditions and heparin treatment are combined, siRNA is released from the PEG-PLL-DMMAn-Mel-siRNA conjugate (Figure 2b, lane 7). In contrast, heparin treatment alone could already unpackage a conventional electrostatic complexed PEG-PLL-DMMAn-Mel/siRNA polyplex (Figure 2b, lane 10).

pH Triggered Lytic Activity of PEG-PLL-DMMAn-Mel-siRNA Conjugates. Erythrocytes were incubated with PEG-PLL-DMMAn-Mel-siRNA conjugates in HBG at pH 7.4 in the absence or presence of serum (Figure 3). The lytic activity of the PEG-PLL-DMMAn-Mel-siRNA conjugate was greatly enhanced after acidic preincubation at pH 5.5, consistent with pH-specific cleavage of the DMMAn groups (Scheme 1b). This pH dependency was not negatively affected by the addition of serum (Figure 3, lower panel, +FCS). In fact, the lytic activity of DMMAn masked conjugates is strongly reduced, while the activity of unmasked conjugates (preincubation at pH 5.5) is largely maintained.

Glutathione Induced Release of siRNA. In the agarose gel electrophoresis study of Figure 2b TCEP was used as reducing agent. Glutathione (GSH) is nature's reducing agent which is found in millimolar concentrations in the cytosol (1−10 mM).[25] In the extracellular environment only micromolar concentrations are present. To clarify if release of siRNA is possible in cells, the siRNA conjugate was incubated at 37 °C with physiological GSH concentrations and release of siRNA was monitored by agarose gel electrophoresis (Figure 4).

Glutathione treatment of 1.25 to 5 mM at 37 °C could induce release of siRNA from the PEG-PLL-DMMAn-Mel-siRNA conjugate (Figure 4, lanes 3−5). Quantification however was not possible, because addition of GSH reduces the EtBr/siRNA fluorescence (see Figure S1 in the Supporting Information).

Gene Knockdown Transfection Results. The biological efficacy of the luciferase siRNA conjugate as well as viability treated cells was evaluated using Neuro2A-eGFPLuc-cells. The covalent conjugate was compared with an analogous electrostatic polyplex formulation (Figure 5).

With the PEG-PLL-DMMAn-Mel/luciferase siRNA polyplex (complexed at w/w ratio 2) a 90% knockdown of luciferase expression could be observed (Figure 5a). The conjugate containing covalently linked GL3 luciferase siRNA enabled 80% luciferase knockdown at lower siRNA doses (0.125 and 0.25 μg) and a 90% knockdown at higher siRNA amounts ($\geq$0.5 μg). With control siRNA polyplexes the luciferase expression was significantly reduced only at the highest siRNA dose (1 μg), indicating some unspecific protein knockdown caused e.g. by carrier toxicity independent from RNAi activity.

This assumption was confirmed by the MTT cytotoxicity assay (Figure 5b). While cells maintained a high metabolic activity at siRNA doses from 0.125 μg to 0.75 μg, the metabolic activity was reduced significantly at the highest applied siRNA dose. In direct comparison, the PEG-PLL-DMMan-Mel-siRNA conjugate showed a reduced cytotoxicity (over 70% metabolic activity after treatment with 1 μg of siRNA in contrast to 50% metabolic activity in case of the polyplex formulation). Please note that the polymer/siRNA (w/w) ratio of the conjugate is 1.6 (molar ratio of PLL:siRNA = 0.68:1; charge ratio $\sim$ 2:1) whereas the ratio is 2 for the polyplex (molar ratio of PLL:siRNA = 0.85:1; charge ratio $\sim$ 3:1). The slight difference in polymer content

(25) Meister, A.; Anderson, M. E. Glutathione. *Annu. Rev. Biochem.* **1983**, *52*, 711–760.

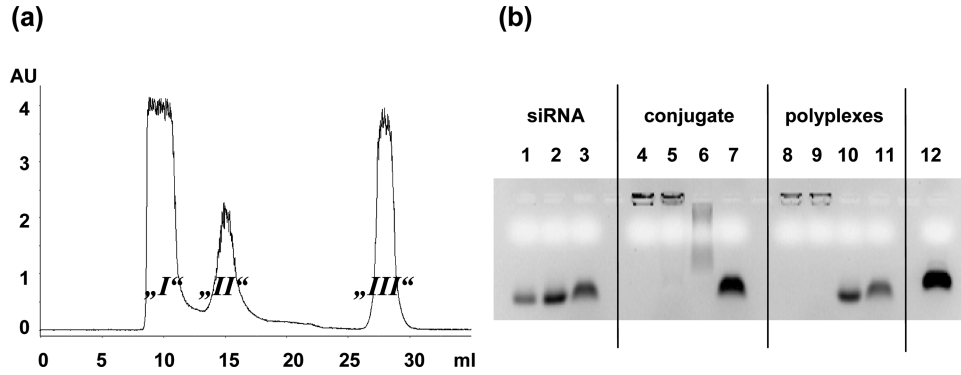


Figure 2. Chromatogram of PEG-PLL-DMMAn-Mel-siRNA conjugate and gel electrophoresis analysis. (a) Purification of the PEG-PLL-DMMAn-Mel-siRNA conjugate. Chromatogram of the mixture containing the siRNA conjugate loaded onto a Superdex 75 HR 10/30 column. The conjugate was eluted with 1 M NaCl, 20 mM Hepes, pH 8.2; flow rate, 0.5 mL/min; *x*-axis, elution volume; *y*-axis, absorption at 280 nm. Peak I, product conjugate; peak II, unbound thiol modified siRNA; peak III, pyridine-2-thione (released from linker during coupling reaction). (b) Agarose gel electrophoresis of purified PEG-PLL-DMMAn-Mel-siRNA conjugate. Lane 1, 0.25 μg of siRNA; lane 2, 0.5 μg of siRNA; lane 3, 0.5 μg of siRNA + heparin + TCEP; lane 4, siRNA conjugate (2.4 μL of peak I, containing 0.5 μg of siRNA); lane 5, peak I + TCEP; lane 6, peak I + heparin; lane 7, peak I + heparin and TCEP; lane 8, PLL/siRNA polyplex (w/w 2/1); lane 9, PLL/siRNA polyplex + TCEP; lane 10, PLL/siRNA polyplex + heparin; lane 11, PLL/siRNA polyplex + heparin and TCEP; lane 12, 20 μL of peak II.

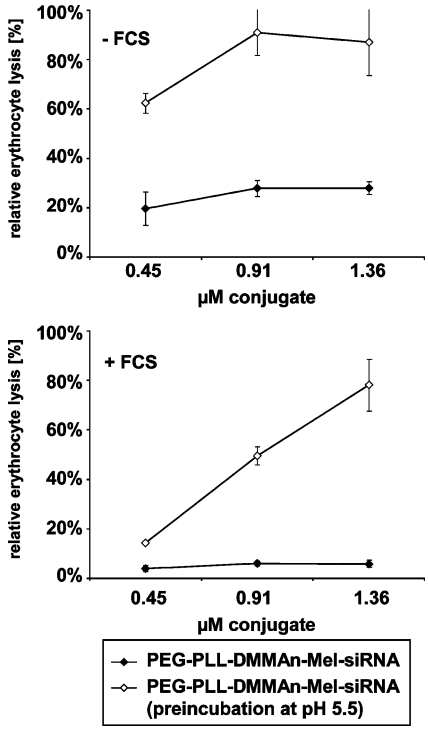


Figure 3. Lytic activities of PEG-PLL-DMMAn-Mel-siRNA conjugates in HBG (upper panel) and in HBG containing 3% FCS (lower panel). Washed murine erythrocytes were incubated with increasing conjugate concentrations. PEG-PLL-DMMAn-Mel-siRNA conjugate was directly applied (◆) or unmasked from DMMAn groups (◇) by preincubation at pH 5.5 for 30 min at room temperature.

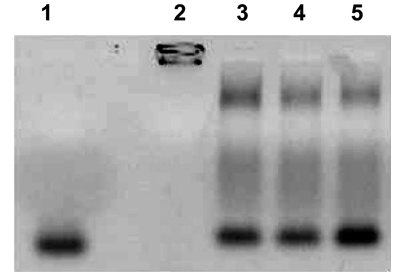


Figure 4. Agarose gel electrophoresis of PEG-PLL-DMMAn-Mel-siRNA conjugate after glutathione treatment. The conjugate was preincubated for 75 min at 37 °C with different amounts of glutathione (GSH). Lane 1, 0.5 μg of siRNA + heparin + GSH (20 mM); lane 2, siRNA conjugate (0.5 μg of siRNA); lane 3, siRNA conjugate + heparin + GSH (1.25 mM); lane 4, siRNA conjugate + heparin + GSH (2.5 mM); lane 5, siRNA conjugate + heparin + GSH (5 mM).

is a plausible explanation for the small differences in knockdown and metabolic activity of cells.

The biological knockdown activity of the covalent luciferase siRNA conjugate was compared side-by-side with the conjugate containing nonsilencing control siRNA over a larger range of siRNA concentrations. Knockdown was observed with doses of ≥31 ng/well (≥25 nM) luciferase siRNA conjugate. Consistent with the concept of specific siRNA knockdown, no reduction of luciferase activity was observed with the control conjugate, unless the high (slightly cytotoxic) 1 μg dose was applied (Figure 6).

Conjugate Aggregation and Particle Sizes. With respect to potential *in vivo* applicability, particle sizes formed by conjugates are of considerable interest. Because size of the formulation can depend on parameters like salt-concentration and/or freeze−thaw processes, size was determined by dynamic light scattering at different formulation steps. Directly after SEC before freezing, the conjugate formed 80 nm (±5 nm) particles in 1 M NaCl. After freezing and thawing, size increased to 120 nm (±10 nm). When this sample was diluted to 0.5 M NaCl and 0.15 M NaCl, sizes

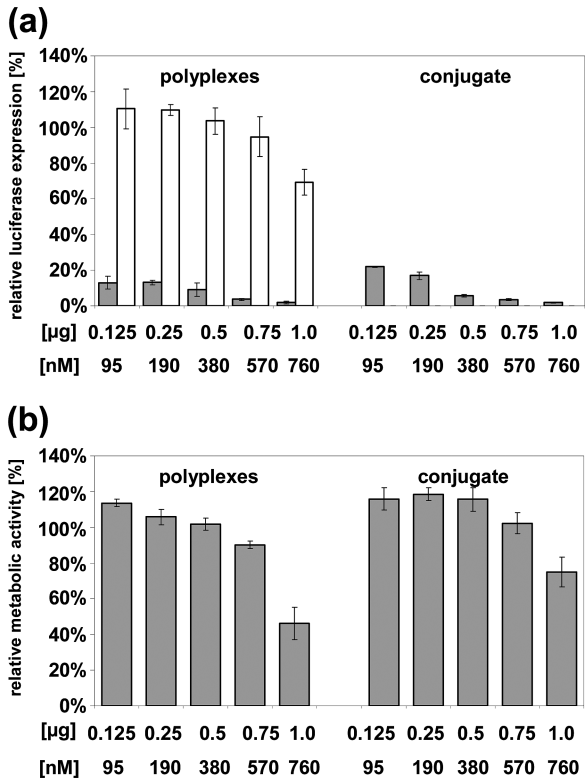


Figure 5. (a) siRNA gene silencing efficiency of PEG-PLL-DMMAn-Mel-siRNA. Luciferase knockdown of the conjugate (right) was compared with knockdown of PEG-PLL-DMMAn-Mel electrostatically complexed at w/w ratio of 2/1 (left). Used amount of siRNA is indicated as [μg] per well and concentration [nM]. Gray bars indicate experiments with luciferase siRNA; white bars indicate control experiments with control siRNA polyplexes. (b) Cell viability after transfection with noncovalent PEG-PLL-DMMAn-Mel/luciferase siRNA polyplexes (left) and the corresponding covalent conjugate formulation (right). Neuro2A-eGFPLuc-cells (5000 seeded cells/well) were treated with formulations containing indicated amounts of siRNA. At 48 h after initial transfection metabolic activity of cells was evaluated by a MTT assay.

grew to 300 and 250 nm, respectively. Dialysis directly after SEC against HBG, snap-freezing and thawing resulted in particles with a hydrodynamic radius of 150 nm ($\pm$5 nm). In summary, the PEG-PLL-DMMAn-Mel-siRNA conjugate formed particles in the range of 80−300 nm, depending on the handling (e.g., freeze thawing, dilution). Smaller, monomolecular structures could not be detected in these DLS experiments. Transmission electron microscopy investigations (see Figure S2 in the Supporting Information) were consistent with the DLS studies, presenting particle sizes of approximately 30−50 nm (width) $\times$ 65−100 nm (length) for siRNA conjugate (formed in 1 M NaCl and diluted in water) similar to the corresponding analogous PEG-PLL-DMMAn-Mel/siRNA polyplexes.

In Vivo **Application and** ***in Vivo*** **Toxicity.** Encouraged by the high *in vitro* bioactivity and biocompatibility, *in vivo* toxicity studies were carried out with PEG-PLL-DMMAn-Mel-siRNA conjugates formulated in osmotically balanced

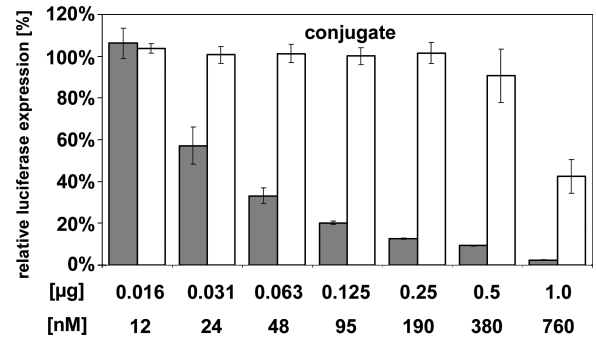


Figure 6. siRNA gene silencing efficiency of PEG-PLL-DMMAn-Mel-siRNA. Luciferase knockdown activity of the siRNA conjugate was compared with knockdown activity of the counterpart containing nonsilencing control siRNA. Used amount of siRNA is indicated as [μg] per well and concentration [nM]. Gray bars indicate transfection with luciferase siRNA conjugates; white bars indicate control siRNA conjugates.

HBG. These experiments displayed an unexpectedly high *in vivo* toxicity of the current formulation. Tumor-free mice tolerated intravenous administration of 12.5 μg of siRNA conjugate, while 25 μg of siRNA conjugate was lethal, with macroscopic lung and liver damage. The same observations were made with two different conjugate batches (synthesized by an initial and an optimized synthetic procedure, see Materials and Methods). Even local application of 25 μg of siRNA conjugate into well-vascularized subcutaneously growing Neuro2A-eGFPLuc tumors in A/J mice showed severe signs of systemic toxicity, like strongly decreased motility and respiratory depression, and mice had to be sacrificed within 10−30 min after application. After opening the abdomen, macroscopic liver damage was observed (bleeding) and blood was also found in the small intestine; other organs like lung or kidney were macroscopically unaffected. The reasons for this toxicity are currently not clear; apparently lack of targeting and/or conjugate *in vivo* aggregation might contribute to this effect. Intravenous application of siRNA-free PEG-PLL-DMMAn-Mel polymer conjugate was found to be far less toxic (data not shown). In any case, formulations have to be improved before further *in vivo* application can be considered.

Discussion

To reach the site of action within the target cell, the nucleic acid has to stay associated with its carrier during the complete extracellular delivery process. Most polyplex and lipoplex formulations are held together electrostatically. While dissociation of polyplexes and release of bioactive therapeutic nucleic acid within the cell is desired, polyplexes can be weakened and be disrupted by other charged biomolecules before they reach the target cell. Burke et al.[19] demonstrated that both the serum and the extracellular matrix can disassemble PEI/DNA polyplexes. PEI/siRNA polyplexes are expected to be even less stable due to the lower number of negative charges per nucleic acid molecule.[21] Reversible

covalent linkage of the delivered nucleic acid to the cationic polymeric carrier may be a way to overcome this drawback.

Recently we described the polymer conjugate PEG-PLL-DMMAn-Mel, which is very effective in complexing and delivering siRNA.[23] Our current work extends this siRNA delivery concept by bioreversible covalent attachment of siRNA to the polymer, characterizing this novel siRNA conjugate (Scheme 1) and comparing it with the noncovalent polyplex analogue. The conjugate contains all bioactive domains (PEG for surface shielding and solubility, PLL as siRNA binding and protecting polycation, the lytic peptide melittin masked by pH-labile DMMAn groups removable at endosomal pH, and siRNA attached via a bioreducible disulfide bond) in one single molecule. Such an all-in-one design guarantees codelivery of all functional domains and circumvents premature disassembly.

To compare the new covalent conjugate with the previously described ionic PEG-PLL-DMMAn-Mel polyplexes,[23] PLL and PEG with the same molecular weights (32 kDa PLL as hydrobromide; 5 kDa PEG) were used. The polycation to siRNA ratio may have an impact on the biophysical and biological properties. Because PEG-PLL-DMMAn-Mel/siRNA polyplexes[23] showed good bioactivity at a PLL/siRNA w/w ratio of 1/1−2/1, it was decided to aim at a similar ratio for the covalent siRNA conjugate. In fact, the synthesis provided a PLL/siRNA w/w ratio of 1.6 within the conjugate. This, taking into account the 5 DMMAn-Mel molecules (=10 negative charges) per siRNA molecule, presents an overall positive/negative charge ratio of 2/1 within the conjugate. A positive charge, on the one hand, is necessary for cell interaction and internalization, because no targeting ligand is included within the conjugate. On the other hand, complexation by the surplus of polycation in addition to the covalent linkage was considered as useful to protect the siRNA against enzymatic degradation. In the current work, PEGylation with 5 kDa PEG was selected for the main purpose to prevent siRNA polyplex aggregation.[23] Perfect shielding was not a focus of this work.

For proper conjugation of the (negatively charged) thiol-modified siRNA with (positively charged) PEG-PLL-PDP by covalent disulfide bond linkage, a key requirement was the use of a high ionic strength buffer; as we previously described,[26] interelectrolyte aggregation can be prevented by such means. After conjugation of siRNA to PEG-PLL-PDP, thiol group containing DMMAn-Mel is added to the reaction mixture for subsequent reaction with PLL via remaining PDP groups. The conjugate was purified by SEC purification (Figure 2a) using an eluent buffer containing a high salt concentration.

The obtained conjugate was characterized by agarose gel electrophoresis, confirming covalent linkage of siRNA and removal of any residual uncoupled free siRNA (Figure 2b). The polyanion heparin disassembles noncovalent siRNA polyplexes, but was unable to dissociate the siRNA conjugate. Heparin was chosen as it is a highly sulfated glycosaminoglycan closely related in structure to heparan sulfates found in the extracellular matrix. The extracellular matrix has been determined to be a significant extracellular barrier to polyplex-mediated *in vivo* gene delivery.[19] Only the combination of heparin plus a reducing agent, such as TCEP (Figure 2b) or the physiologically more relevant glutathione (Figure 4), was able to release the siRNA from the conjugate. Though we do not know formally whether disulfide cleavage is required for the biological activity of PEG-PLL-DMMAn-Mel-siRNA, literature indicates a possible advantage of bioreducibility. Derfus et al.[27] synthesized quantum dots containing covalently bound siRNA. Reversibly attached siRNA by disulfide cross-linkers showed greater silencing efficiency than siRNA attached by a nonreducible thioether linkage, which also suggests that disulfide cleavage in the intracellular takes place and that release of the siRNA is advantageous.[27] Similar observations were made by Rozema et al.,[14] who also found that a bioreversible siRNA linkage is advantageous in terms of silencing activity. A disulfide trigger has also been used in other steps for enhancing transfer of therapeutic nucleic acids, such as to

(26) Wagner, E.; Plank, C.; Zatloukal, K.; Cotten, M.; Birnstiel, M. L. Influenza Virus Hemagglutinin HA-2 N-Terminal Fusogenic Peptides Augment Gene Transfer by Transferrin-Polylysine-DNA Complexes: Toward a Synthetic Virus-Like Gene-Transfer Vehicle. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7934–7938.

(27) Derfus, A. M.; Chen, A. A.; Min, D. H.; Ruoslahti, E.; Bhatia, S. N. Targeted Quantum Dot Conjugates for siRNA Delivery. *Bioconjugate Chem.* **2007**, *18*, 1391–1396.

(28) Saito, G.; Amidon, G. L.; Lee, K. D. Enhanced Cytosolic Delivery of Plasmid DNA by a Sulfhydryl-Activatable Listeriolysin O/Protamine Conjugate Utilizing Cellular Reducing Potential. *Gene Ther.* **2003**, *10*, 72–83.

(29) Choi, S.; Lee, K. D. Enhanced Gene Delivery Using Disulfide-Crosslinked Low Molecular Weight Polyethylenimine With Listeriolysin O-Polyethylenimine Disulfide Conjugate. *J. Controlled Release* **2008**, *131*, 70–76.

(30) Chen, C. P.; Kim, J. S.; Steenblock, E.; Liu, D.; Rice, K. G. Gene Transfer With Poly-Melittin Peptides. *Bioconjugate Chem.* **2006**, *17*, 1057–1062.

(31) Christensen, L. V.; Chang, C. W.; Kim, W. J.; Kim, S. W.; Zhong, Z.; Lin, C.; Engbersen, J. F.; Feijen, J. Reducible Poly(Amido Ethylenimine)s Designed for Triggered Intracellular Gene Delivery. *Bioconjugate Chem.* **2006**, *17*, 1233–1240.

(32) Breunig, M.; Lungwitz, U.; Liebl, R.; Goepferich, A. Breaking Up the Correlation Between Efficacy and Toxicity for Nonviral Gene Delivery. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 14454–14459.

(33) Lee, Y.; Mo, H.; Koo, H.; Park, J. Y.; Cho, M. Y.; Jin, G. W.; Park, J. S. Visualization of the Degradation of a Disulfide Polymer, Linear Poly(Ethylenimine Sulfide), for Gene Delivery. *Bioconjugate Chem.* **2007**, *18*, 13–18.

(34) Wang, X. L.; Jensen, R.; Lu, Z. R. A Novel Environment-Sensitive Biodegradable Polydisulfide With Protonatable Pendants for Nucleic Acid Delivery. *J. Controlled Release* **2007**, *120*, 250–258.

(35) Lin, C.; Zhong, Z.; Lok, M. C.; Jiang, X.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. Novel Bioreducible Poly(Amido Amine)s for Highly Efficient Gene Delivery. *Bioconjugate Chem.* **2007**, *18*, 138–145.

activate endosomolytic proteins[28,29] or peptides,[30] to enable intracellular biodegradation[31−35] or deshielding of the carrier.[36,37]

The expected pH responsiveness of the PEG-PLL-DMMAn-Mel-siRNA conjugate was proven in an erythrocyte leakage assay (Figure 3). Consistent with the acidic cleavage of the DMMAn masking groups from melittin, incubation of the conjugate at endosomal pH 5.5 strongly enhanced the lytic activity.

The gene silencing potential of PEG-PLL-DMMAn-Mel-siRNA was evaluated using cultured Neuro2A cells stably expressing luciferase. High gene knockdown activity (Figure 5a) was observed also at low doses (around 0.1 μg of siRNA/well). A comparable knockdown was seen with the electrostatic PEG-PLL-DMMAn-Mel polyplexes formed at w/w 2 polyplexes, indicating that covalent attachment of siRNA did not weaken the siRNA delivery efficiency of the polycationic carrier. *In vitro* carrier toxicity was very low and detectable only at the highest dose of 1 μg of siRNA/well (Figure 5b). Transfection with a conjugate containing covalently attached control siRNA did not affect luciferase expression at siRNA doses of up to 0.75 μg, indicating a specific RNAi-based knockdown (Figure 6).

In summary, the bioresponsive endosomolytic PEG-PLL-DMMAn-Mel-siRNA conjugate belongs to a new generation of dynamic, multifunctional nucleic acid carriers which are programmed to undergo molecular changes triggered by the physiological environment.[28,36−41] Covalent attachment of siRNA[42] may provide several advantages; as shown here, it improves stability of the siRNA formulation against natural occurring polyanions like heparin. The endosomal pH is expected to recover the lytic form of melittin required for release from endosome; the reducing intracellular environment may contribute to optimum siRNA release within the cytosol. Consistently, the conjugate shows excellent *in vitro* knockdown activity comparable to the electrostatically formed siRNA complex. However, despite the very encouraging bioactivity and good biocompatibility *in vitro*, intravenous and intratumoral *in vivo* applications in mice showed unexpectedly high toxicity with the current formulation. The reasons for this are currently not clear; the current conjugates were however designed primarily for *in vitro* comparison with the noncovalent polyplex,[23] and lack of targeting, incomplete PEG shielding and conjugate aggregation might contribute to the *in vivo* toxicity. Therefore, to exploit the full potential of the concept and enable *in vivo* application, novel siRNA conjugates with improved shielding and targeting ligands remain to be synthesized and tested *in vivo*.

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Supporting Information Available: Additional experimental details, Figure S1 depicting the influence of GSH concentration on EtBr-siRNA fluorescence, and Figure S2 depicting TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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(36) Takae, S.; Miyata, K.; Oba, M.; Ishii, T.; Nishiyama, N.; Itaka, K.; Yamasaki, Y.; Koyama, H.; Kataoka, K. PEG-Detachable Polyplex Micelles Based on Disulfide-Linked Block Catiomers As Bioresponsive Nonviral Gene Vectors. *J. Am. Chem. Soc.* **2008**, *130*, 6001–6009.

(37) Kim, S. H.; Jeong, J. H.; Lee, S. H.; Kim, S. W.; Park, T. G. PEG Conjugated VEGF siRNA for Anti-Angiogenic Gene Therapy. *J. Controlled Release* **2006**, *116*, 123–129.

(38) Murthy, N.; Campbell, J.; Fausto, N.; Hoffman, A. S.; Stayton, P. S. Bioinspired PH-Responsive Polymers for the Intracellular Delivery of Biomolecular Drugs. *Bioconjugate Chem.* **2003**, *14*, 412–419.

(39) Sethuraman, V. A.; Na, K.; Bae, Y. H. pH-Responsive Sulfonamide/PEI System for Tumor Specific Gene Delivery: an in vitro Study. *Biomacromolecules* **2006**, *7*, 64–70.

(40) Wagner, E. Programmed Drug Delivery: Nanosystems for Tumor Targeting. *Expert. Opin. Biol. Ther.* **2007**, *7*, 587–593.

(41) Wolff, J. A.; Rozema, D. B. Breaking the Bonds: Non-Viral Vectors Become Chemically Dynamic. *Mol. Ther.* **2008**, *16*, 8–15.

(42) Jeong, J. H.; Mok, H.; Oh, Y. K.; Park, T. G. siRNA Conjugate Delivery Systems. *Bioconjugate Chem.* **2009**, *20*, 5–14.