

# Optimized polyethylenimine (PEI)-based nanoparticles for siRNA delivery, analyzed in vitro and in an ex vivo tumor tissue slice culture model

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**Abstract** The non-viral delivery of small RNA molecules like siRNAs still poses a major bottleneck for their successful application in vivo. This is particularly true with regard to crossing physiological barriers upon systemic administration. We have previously established polyethylenimine (PEI)-based complexes for therapeutic RNA formulation. These nanoplexes mediate full RNA protection against nucleolytic degradation, delivery to target tissues as well as cellular uptake, intracellular release and therapeutic efficacy in preclinical in vivo models. We herein present data on different polyplex modifications for the defined improvement of physicochemical and biological nanoparticle properties and for targeted delivery. (i) By non-covalent modifications of PEI polyplexes with phospholipid liposomes, ternary complexes (“lipopolyplexes”) are obtained that combine the favorable features of PEI and lipid systems. Decreased cytotoxicity and highly efficient delivery of siRNA is achieved. Some

lipopolyplexes also allow prolonged storage, thus providing formulations with higher stability. (ii) Novel tyrosine modifications of low molecular weight PEI offer further improvement of stability, biocompatibility, and knockdown efficacy of resulting nanoparticles. (iii) For ligand-mediated uptake, the shielding of surface charges is a critical requirement. This is achieved by PEI grafting with polyethylene glycol (PEG), prior to covalent coupling of anti-HER1 antibodies (Erbix®) as ligand for targeted delivery and uptake. Beyond tumor cell culture, analyses are extended towards tumor slice cultures from tumor xenograft tissues which reflect more realistically the in vivo situation. The determination of siRNA-mediated knockdown of endogenous target genes, i.e., the oncogenic survival factor survivin and the oncogenic receptor tyrosine kinase HER2, reveals nanoparticle penetration and biological efficacy also under intact tissue and stroma conditions.

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## Introduction

By silencing any target gene of interest through sequence-specific mRNA cleavage, RNA interference (RNAi) provides a potent strategy for the treatment of various diseases. Of critical importance for RNAi induction, however, is the delivery of small interfering RNA (siRNA), especially when it comes to their in vivo applications. Delivery systems need to address the otherwise rapid nuclease degradation and renal elimination upon systemic administration of the siRNA, poor cellular uptake due its high anionic charge density and

insufficient lysosomal release. So far, these issues still pose major hurdles for translating siRNA into the clinic [1, 2].

Non-viral vectors for siRNA delivery can be based on cationic compounds such as lipids or polymers, allowing for encapsulation or complexation of siRNAs in nano-sized particles [3–7]. Important biological parameters are complex/nanoparticle stability in the presence of body fluids, cellular uptake and transfection efficacy, biocompatibility and, *in vivo*, favorable pharmacokinetic properties and bioavailability [8–10].

Polyethylenimines (PEIs) are water-soluble linear or branched polymers with a broad range of molecular weights (0.8–1000 kDa). They have been widely explored for the delivery of nucleic acids *in vitro* and *in vivo* [11–14]. Due to the presence of a large number of partially protonated amino groups, PEIs are able to efficiently condense nucleic acids, thereby preventing enzymatic digestion and protecting them against the endosomal/lysosomal degradation [15–17]. Due to the so-called “proton sponge effect,” the high buffer capacity of PEI also triggers lysosomal escape [18]. The 25 kDa branched PEI is considered as the “gold standard” for transfection; however, while transfection efficacy positively correlates with molecular weight, this also increases cytotoxicity [16, 19]. Consequently, lower molecular weight linear or branched PEIs have been described for *in vitro* and *in vivo* use [20, 21]. These also include the 4–12 kDa branched PEI F25-LMW for *in vitro* and *in vivo* delivery of small RNA molecules [17, 22, 23].

For developing more efficient and less toxic PEI derivatives, several strategies have been explored. Among others, these include the preparation of ternary complexes by mixing pre-formed PEI complexes with liposomes ([24, 25], Ewe et al., pending revisions), amino acid modification of PEI or the covalent coupling of antibodies for targeted delivery.

The non-covalent combination of cationic polymer-based complexes with lipids, leading to so-called lipopolyplexes, has been explored for DNA delivery and found to improve various physicochemical and biological parameters [26–31]. Lipopolyplexes containing the low molecular weight PEI F25-LMW and the phospholipid DPPC, with or without different co-lipids, have been described for the delivery of siRNA. They showed very good transfection efficiencies despite strongly reduced surface charges as well as improved biocompatibility and excellent storage stabilities under various conditions [25, 32]. Since phospholipids are the main components of cell membranes, they are associated with high biocompatibility and clinically approved for drug delivery [33, 34]. In this paper, our previous studies are further extended towards different serum stabilities of various lipopolyplex formulations and the preservation of their biological activities upon prolonged storage under physiological conditions.

The modification of PEI with arginine, lysine, and leucine has been tested previously for plasmid DNA delivery, yielding

enhanced *in vitro* transfection efficacies particularly for the cationic amino acids lysine and arginine [35]. In another study exploring the hydrophobic amino acids tryptophan, leucine, phenylalanine or tyrosine linked to branched 25 kDa PEI, the tyrosine-modified 25 kDa PEI was identified as the most potent PEI derivative in tissue culture [36]. This more efficient siRNA delivery was linked to polymer self-assembly and improved proton sponge properties [37]. Beyond 25 kDa PEI which is generally considered as relatively toxic due to its molecular weight, we have recently extended the approach of tyrosine modification towards a lower molecular weight 10 kDa PEI [38]. In the present paper, we proceed even further by including 5 and 2 kDa PEI, aiming at further enhanced biocompatibility and efficacy.

To improve target organ/target cell specificity, ligand-mediated binding and uptake strategies have been explored for various nanoparticles (see, e.g. [39, 40] for current reviews). However, the identification of optimal ligands for cell-specific binding, its presentation on the nanoparticle surface in a way that binding affinity is preserved, and the reduction of non-specific nanoparticle interactions with non-target tissues are major issues. Antibody-antigen binding affinities are among the strongest protein-protein interactions observed in nature. Targeted delivery of modified PEI complexes has been explored as well [41, 42]. The coupling of antibodies directed, e.g., against receptor tyrosine kinases overexpressed on tumor cells, e.g., the EGF receptor EGFR (HER1), is a promising approach in the development of targeted nanoparticle delivery. We have previously described antibody-modified, PEGylated PEI conjugates, generated by chemical coupling of the anti-EGFR antibody cetuximab, to PEI via PEG spacer molecules, and analyzed their binding properties in surface plasmon resonance (SPR) experiments [43]. In this paper, we explore their biological efficacies in tissue culture and in an *ex vivo* tissue slice model.

Beyond the use of organotypic rodent brain slice cultures in neuroscience (see, e.g. [44]), this model has been also explored with regard to tumor-derived slice cultures. They offer a powerful tool for determining tissue responses to various stimuli [45, 46], and in particular, allow for studying tumor cells in their intact environment, including tumor stroma. This also offers an approach for the direct administration of nanoparticles for pharmacological or toxicological analyses, which however has been barely explored so far [47, 48]. Recently, we have established tissue slice cultures from tumor xenografts as an analysis platform for the systematic nanoparticle assessment regarding siRNA-mediated knockdown efficacies and tissue penetration (Merz et al., pending revisions). In the present paper, we show a very profound PEI/siRNA-mediated knockdown of important oncogenes and extend our studies towards the use of antibody-conjugated complexes for targeted delivery.

## Materials and methods

Branched polyethylenimines 2 and 25 kDa were obtained from Sigma-Aldrich (Taufkirchen, Germany), 10 kDa PEI was from Polysciences (Eppelheim, Germany) and 5 kDa PEI is a kind gift from BASF SE (Ludwigshafen, Germany). The branched PEI F25-LMW (4–12 kDa) was prepared as previously described [22]. The lipids dipalmitoyl-phosphatidyl-choline (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol (DPPG), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(tert-Butoxycarbonyl)-L-tyrosine (Boc-Tyrosine) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC · HCl) were purchased from Carbolution Chemicals (Saarbrücken, Germany), *N*-hydroxysuccinimide (NHS) from Thermo Scientific Pierce Protein Biology (Schwerte, Germany), and trifluoroacetic acid (TFA) from Carl Roth (Karlsruhe, Germany). Organic solvents were of HPLC grade and purchased from different vendors, and dichloromethane (DCM) was further dried over activated molecular sieves (3 Å). Dry dimethylformamide (DMF) was from VWR (Darmstadt, Germany). Wild-type cell lines were obtained from the American type culture collection (ATCC; Manassas, VA). The stable luciferase-expressing cell line SKOV-3-Luc cell line was described previously [49]. Cell culture media, Dulbecco's phosphate buffered saline (PBS), and trypsin were from Sigma-Aldrich, and fetal calf serum (FCS) was purchased from Gibco Thermo Fisher (Darmstadt, Germany). Cell culture plastic and other disposable plastics were from Sarstedt (Nümbrecht, Germany).

Chemically synthesized siRNAs were purchased from MWG (Ebersberg, Germany) or Dharmacon/GE Healthcare (Lafayette, CO), with sequences as follows: siCtrl. (siLuc2, serving as negative control): sense: 'CGUACGCGGAAUACUUCGATTdTdT-3'; antisense: 3'-dTdTGCAUGCGCCUUAUGAAGCU-5'; siLuc; sense: 5'-CUUACGCUGAGUACUUCGAdTdT-3', antisense: 3'-dTdTGAAUGC GACUCAUGAAGCU-5'; siHER2; sense: 5'-GCCUGAAUAUGUGAACAGdTdT-3'; antisense: 5'-CUGGUUCACAUUUCAGGCdTdT-3'; siSurvivin: sense: 5'-GAAUUAACCCUUGGUGAAUdTdT-3', antisense: 5'-AUUACCAAGGGUUAUUCdTdT-3'. Scrambled Alexa647 fluorophore-labeled siRNA was purchased from Qiagen (Hilden, Germany). All other standard chemicals were of analytical grade.

### Chemical synthesis of tyrosine-modified PEI and PEG grafted/Erbbitux functionalized PEI

For tyrosine modification of PEI, *N*-Boc-tyrosine (0.65 g, 2.3 mmol) and *N*-hydroxysuccinimide (0.27 g, 2.3 mmol) were dissolved in 3 mL DMF/DCM (1:1 v/v) under a nitrogen atmosphere, prior to adding EDC · HCl (0.45 g, 2.3 mmol) and stirring for 4 h at RT. In a round-bottom flask, the respective

branched PEI (0.2 g, 4.65 mmol in ethylenimine) was dissolved in 4 mL DMF/DCM (1:1 v/v) under a nitrogen atmosphere. The pre-activated tyrosine mixture was then added to the polymer solution and allowed to proceed for 3 days at RT. In vacuo, volatile solvents were removed prior to precipitating the crude polymer in diethyl ether. The residue was dissolved in 5 mL DCM and 5 mL TFA, and stirred for 3 h for Boc-deprotection prior to removing the solvent under reduced pressure and removing excess TFA by co-evaporation with ethanol (3 × 50 mL). The product was dissolved in 0.1 M HCl and excessively purified by dialysis (MWCO 3500 Da, SpectraPor) against water for 3 days. Lyophilization (Christ, Osterode, Germany) yielded the respective tyrosine-modified PEI as a yellowish powder. The degree of functionalization was confirmed by <sup>1</sup>H-NMR in D<sub>2</sub>O (Mercury plus, 300 MHz, Varian Agilent Technologies, Santa Clara, CA).

PEG-PEI was synthesized by PEGylation of PEI F25-LMW using MS(PEG)<sub>24</sub>, and PEG-PEI-Erbbitux conjugates were prepared from this PEG-PEI by covalent coupling of the antibody to PEI molecules using the heterobifunctional PEG linker SM(PEG)<sub>24</sub>, both as described previously [43].

### Liposome, PEI complex, and lipopolyplex preparation

The liposomes DPPC, DPPC/DPPE (85:15 mol/mol), DPPC/DPPG (92:8 mol/mol) were prepared by the hydration/extrusion method as described previously [25]. Five milligram lipid(s) in chloroform/methanol (2:1, v/v) were dried in a round-bottom flask and hydrated with 1 mL sterile dH<sub>2</sub>O. The suspension was incubated at 55 °C in an ultrasound bath sonicator for a few minutes. To form unilamellar liposomes, the suspension was finally extruded 11 times through a 200-nm polycarbonate membrane in a Mini-Extruder at 45 °C (Avanti Polar Lipids).

PEI/siRNA complexes were prepared as described [22]. Briefly, the desired amount of siRNA was complexed with the different PEIs at mass ratios 7.5 (PEI F25-LMW) or as indicated in the figures. For example, for a 24-well 60 pmol/0.8 µg and the appropriate amount of PEI were each diluted in 12.5 µL HN buffer (150 mM NaCl, 10 mM HEPES, pH 7.4). Two- and five-kilodalton tyrosine-PEIs were diluted in 5 % glucose solution. The PEI solution was added to the siRNA solution, vortexed, and incubated for 30 min at room temperature. The lipopolyplexes were prepared by incubating equal volumes of PEI/siRNA complexes with liposomes at a PEI/lipid mass ratio of 5 [25]. To this end, 25 µL polyplex containing 4 µg PEI F25 and 25 µL DPPC liposomes comprising 20 µg lipid were properly mixed by pipetting and vortexing, and incubated for at least 1 h at room temperature prior to use. For evaluating the influence of FCS on biological activities, polyplexes or lipopolyplexes were incubated in the presence or absence of FCS as indicated in the figures.

### Measurement of complex sizes, stabilities and zeta potentials, and complexation efficacies

The hydrodynamic diameters and zeta potentials of siRNA complexes were measured with a Brookhaven ZetaPALS system (Brookhaven Instruments, Holtsville, NY) according to [25]. Complexation efficacies were determined according to [38], and complex stabilities were analyzed essentially as described in [50], but without prior radioactive siRNA labeling. siRNA of 0.2  $\mu\text{g}$  measurement was utilized, with subsequent analysis of free siRNA by agarose gel electrophoresis.

### Cell culture, transfections, and assays

All cell lines were cultured in a humid atmosphere at 37 °C and 5 % CO<sub>2</sub>. SKOV-3, SKOV-3-Luc, PC-3, A431, and U87 were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM alanyl-glutamine and 10 % FCS. Standard transfections were performed in 24-well plates. The day before, cells were seeded at a density of 30,000 cells per well and 1 mL (or 0.5 mL in the case of tyrosine-modified PEIs) fully supplemented medium. For RT-qPCR experiments, 100,000 cells were seeded in 6-well plates and 2 mL cell culture medium. Usually, no further medium change was done after the polyplexes or lipopolyplexes were added (for amounts used for transfection, see figure legends).

The determination of acute cell damage upon the transfection was performed by measuring the lactate dehydrogenase (LDH) release with the Cytotoxicity Detection Kit from Roche (Mannheim, Germany) according to the manufacturer's protocol and as described previously [51].

Luciferase activities were determined 72 h post transfection using the Beetle-Juice Kit (PJK, Kleinblittersdorf, Germany). The medium was aspirated prior to adding 100  $\mu\text{L}$  lysis buffer (Promega, Mannheim, Germany) and incubation for 30 min at RT. In a test tube, 25  $\mu\text{L}$  luciferin substrate was mixed with 10  $\mu\text{L}$  cell lysate and luminescence was immediately measured in a luminometer (Berthold, Bad Wildbad, Germany).

Total RNA was extracted after 96 h using the peqGOLD Trifast reagent (Peqlab, Erlangen, Germany) following the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop 2000c (Thermo Fisher, Schwerte, Germany). The determination of endogenous target gene mRNA levels was performed by RT-qPCR, essentially as described previously [52], with the following primer sequences: house keeper  $\beta$ -actin for: 5'CCAACCGCGAGAAGATGA-3', rev: 5'-CCAGAGGCGTACAGGGATAG-3'; survivin for: 5'-TGATGAGAGAATGGAGACAGAG-3', rev: 5'-ACAGCAGTGGCAAAGGAG-3'; HER2 for: 5'-TGGCTCAGTGACCTGTTTTG-3', rev: 5'-GGTCCTTAGTGGGCACAGG-3'.

### Tissue slice preparation, cultivation, and treatment

For tissue slice preparation, subcutaneous tumor xenografts generated in athymic nude mice (CrI:NU-Foxn1nu, Charles River Laboratories, Sulzfeld, Germany) were explanted and subjected to sectioning, essentially as described previously ([45], Merz et al., pending revisions). Briefly, the tumor tissue was cut with an autoclaved standard razor blade into 5-mm cubes. These were placed on a pile of sterile filter membranes soaked with the preparation medium, and fixated by Histoacryl glue (Braun, Melsungen, Germany) to facilitate the cutting procedure. Three-hundred-fifty-micrometer slices were prepared by a tissue chopper (McIlwain TC752), placed in a container with preparation medium, and then transferred in groups of three onto membrane culture inserts, prior to cultivation in 6-well plates filled with 1 ml cultivation medium per well. The cultivation medium was composed of MEM (Gibco Thermo Fisher), 25 % Hank's Balanced Salt Solution (with Ca and Mg; Gibco), 10 % normal horse serum (Gibco), 1 % penicillin/streptomycin, 1 % L-glutamine, and 1 % glucose. The plates were kept in a humidified incubator at 37 °C and 5 % CO<sub>2</sub> with the cultivation medium being replaced every day. For transfection, 75  $\mu\text{L}$  of the above polyplexes (~3 drops per slice) were applied once onto the slices prior to cultivation until harvesting.

## Results and discussion

### Preservation of biological efficacies of various lipopolyplexes upon storage in the presence of serum

By combining PEI F25-LMW/siRNA complexes with liposomes (DPPC, DPPC/DPPE, or DPPC/DPPG), lipopolyplexes were formed [24, 25]. Lipopolyplexes were analyzed for size and zeta potential and compared to the parent PEI/siRNA complexes (Suppl. Table 1). With the exception of DPPC, effective diameters increased upon lipopolyplex formation, indicative of complex incorporation into liposomes or at least the association of the lipids on the outer complex surface. This notion is also supported by electron microscopy demonstrating complex incorporation into even-shaped liposome-like structures (data not shown), inhibition of aggregation (see below) and marked alterations in the complex zeta potential upon lipopolyplex formation, due to the introduction of oppositely charged lipids (Suppl. Table 1). In heparin displacement assays, however, complex stabilities were found unchanged between PEI/siRNA complexes and the corresponding DPPC/PEI/siRNA lipopolyplexes (Suppl. Fig. 1a), indicating that this outer lipid shell does not lead to further protection.

For the determination of biological efficacies, stably luciferase-expressing SKOV-3 ovarian carcinoma cells

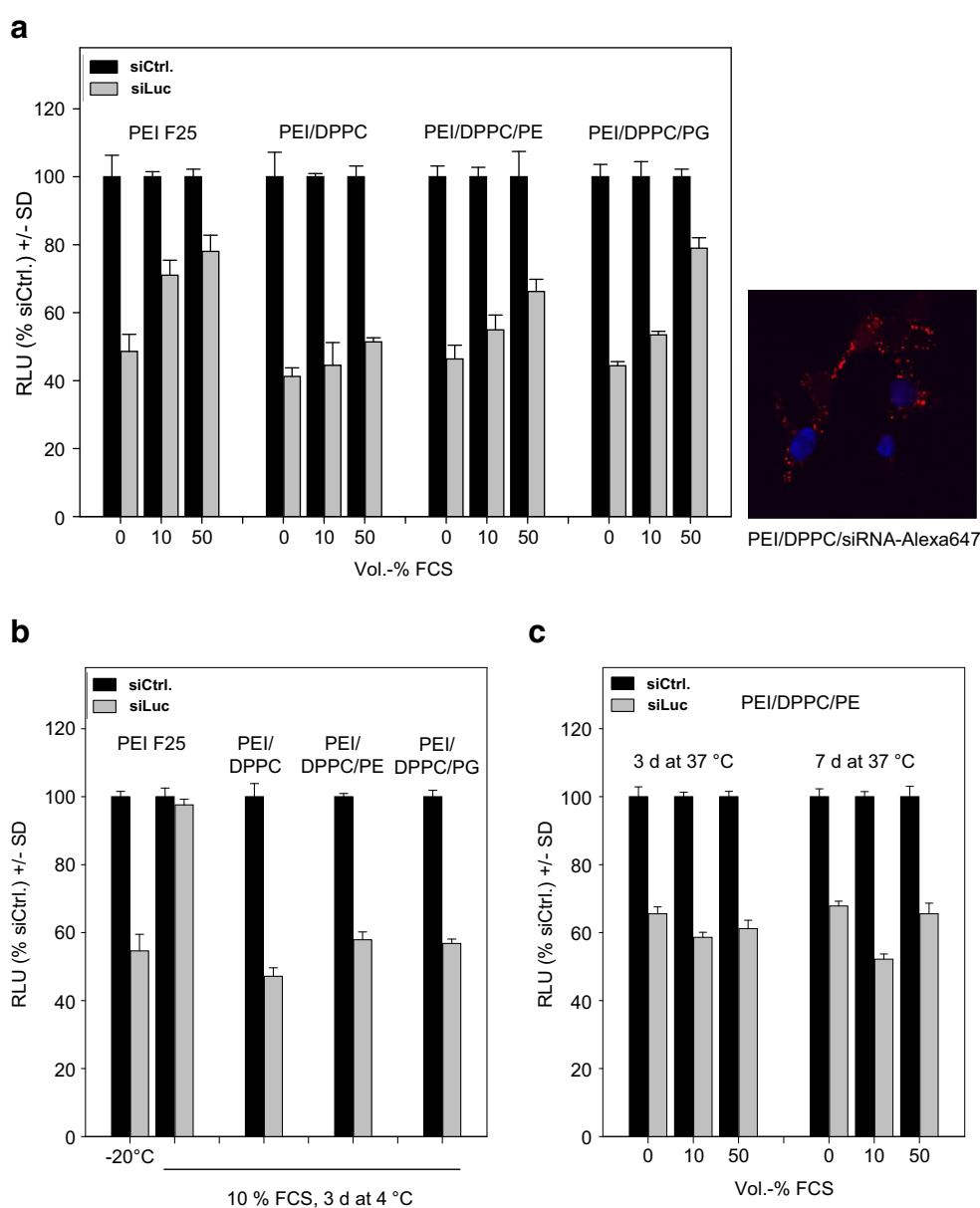


(SKOV-3-Luc) were transfected with polyplexes or lipopolyplexes containing a luciferase-specific siRNA (siLuc) or a negative control siRNA (siCtrl.) to exclude non-specific effects. In the case of PEI/siRNA complexes, a ~50 % knock-down was observed in the absence of serum, which decreased with increasing concentrations of fetal calf serum (FCS; Fig. 1a). A similar trend was observed for PEI/DPPC/DPPG/siRNA lipopolyplexes. Quite in contrast, lipopolyplexes containing DPPC or DPPC/DPPE led to the protection of knock-down efficacy (Fig. 1a, center panels). Confocal microscopy using Alexa647-labeled siRNA also revealed efficient cellular uptake of the PEI/DPPC/siRNA lipopolyplexes as necessary prerequisite for gene knockdown (Fig. 1a, right).

A major issue in the case of many polymeric nanoparticles including PEI/siRNA complexes is their colloidal

instability, i.e., their tendency to aggregate upon storage [53–55]. While we have shown previously that serum actually can inhibit this effect to a certain extent [25], still a total loss of knockdown efficacy was observed upon storage of PEI/siRNA complexes for 3 days at 4 °C (Fig. 1b, left). In contrast, all lipopolyplexes retained their biological activity after being stored under the same conditions, indicating protection of colloidal stability (Fig. 1b). Beyond previous studies [25], we demonstrate here that this is true for several lipopolyplexes with different lipid compositions. This protective effect was also seen when switching to more important physiological conditions, i.e., 37 °C and the presence of serum. More specifically, a ~40 % knockdown efficacy of PEI/DPPC/DPPE/siRNA lipopolyplexes was fully retained over up to 7 days (Fig. 1c).

**Fig. 1** **a** Knockdown efficacies of various fresh lipopolyplexes (60 pmol siRNA) in the presence of the indicated serum concentrations after 1 h incubation, or upon their prolonged storage **b** at 4 °C or **c** at 37 °C, as determined by luciferase expression levels in stably luciferase-expressing SKOV-3-Luc cells. PE = DPPE; PG = DPPG. Confocal microscopy also shows the uptake of PEI/DPPC/siRNA lipopolyplexes; *red*: Alexa647-labeled siRNA; *blue*: cell nuclei (DAPI). **e** Structure formula of tyrosine-modified PEI

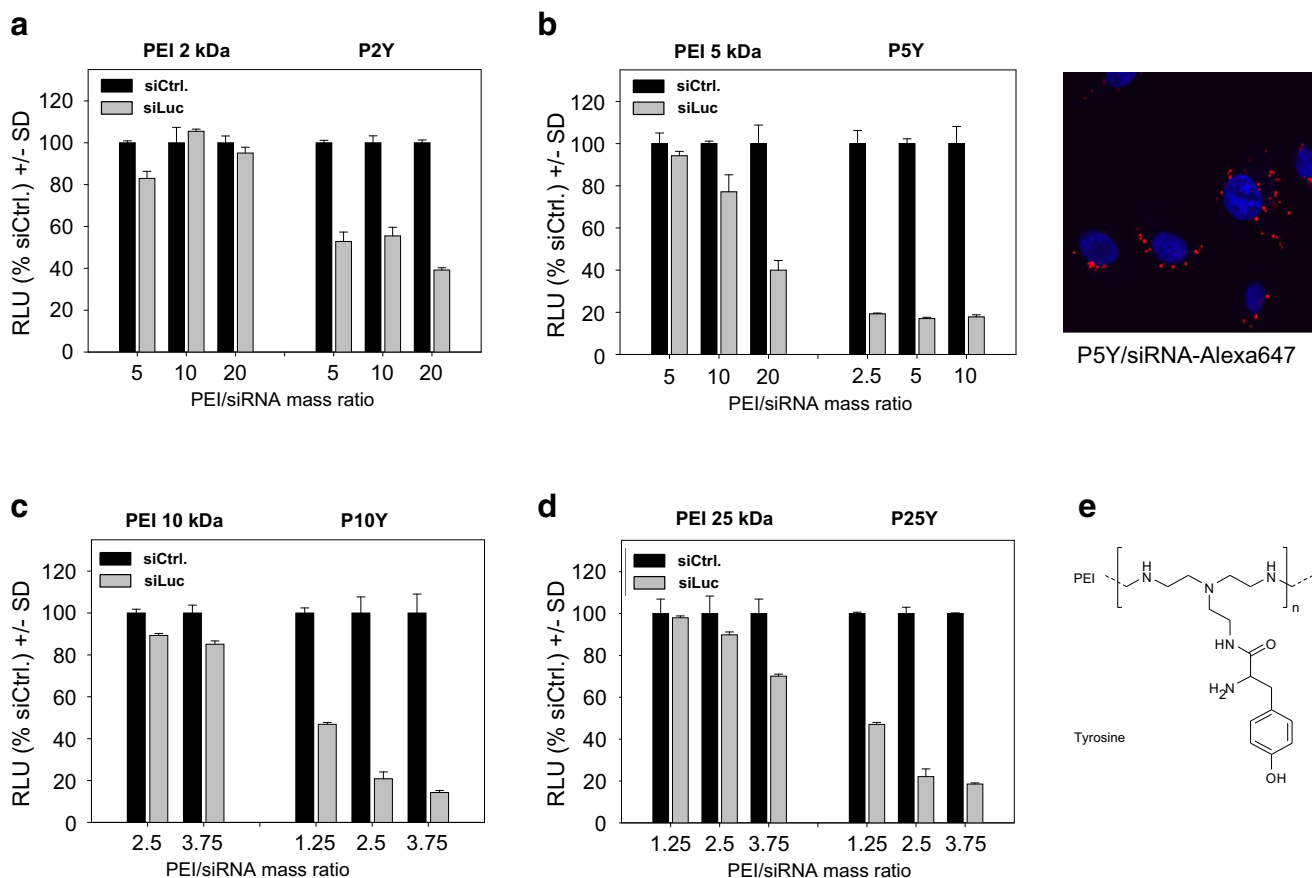


Taken together, we conclude that lipopolyplex formation leads to enhanced knockdown efficacy under serum conditions and allows for the prolonged storage of the nanoparticles even at 37 °C without aggregation.

### Tyrosine modification of PEI for improved bioactivity

It has been shown previously that the covalent coupling of tyrosine to 25 kDa branched PEI may lead to more efficient siRNA delivery [36], based on polymer self-assembly and improved proton sponge properties [37]. We have extended this approach towards lower molecular weight PEIs due to their higher biocompatibility as compared to 25 kDa PEI. More specifically, branched 2 kDa, 5 kDa, 10 kDa and, for comparison, 25 kDa PEI were tyrosine modified as described previously [36, 38], leading to covalent binding of tyrosine preferentially to primary amines (see Fig. 2e for structure). To this end, *N*-Boc protected tyrosine was activated with NHS and EDC, prior to coupling to PEI nitrogens. In <sup>1</sup>H-NMR analysis of the tyrosine-modified PEIs (termed “PxY” with *x* = molecular weight of the parent PEI), a ~30 % degree of

substitution was found (data not shown). In particular, for the very low molecular weight PEIs, which may complex siRNAs only poorly, PEI/siRNA mass ratios were defined which mediated efficient complexation (Suppl. Fig. 1b). More specifically, a polymer/siRNA mass ratio 1.25 led to partial complexation, while siRNA was completely complexed at ratio 2.5, as indicated by the absence of free siRNA bands. Only the tyrosine-modified PEI with the lowest molecular weight, P2Y, required a mass ratio 5 for complete complexation (Suppl. Fig. 1b, left). Complexes were determined by photon correlation spectroscopy (PCS) to be in the range of 300–400 nm (Suppl. Table 1). In the case of the low molecular weight 2 and 5 kDa PEIs, a trend towards larger complex sizes upon tyrosine modification was observed, which was not so in the case of P10Y and P25Y. Simultaneously, a decrease in zeta potential from ~30 mV to ~15–20 mV was observed upon tyrosine coupling, independent of the molecular weight of PEI (Suppl. Table 1). The notion of enhanced complex stability upon tyrosine modification was also supported by heparin displacement assays. Despite lower PEI/siRNA mass ratios used for complexation,



**Fig. 2** Analysis of various tyrosine-modified PEIs, as compared to their respective non-modified counterparts, with regard to luciferase knockdown efficacies in stably luciferase-expressing SKOV-3-Luc cells. Different PEI/siRNA mass ratios (30 pmol/0.4 μg siRNA) were

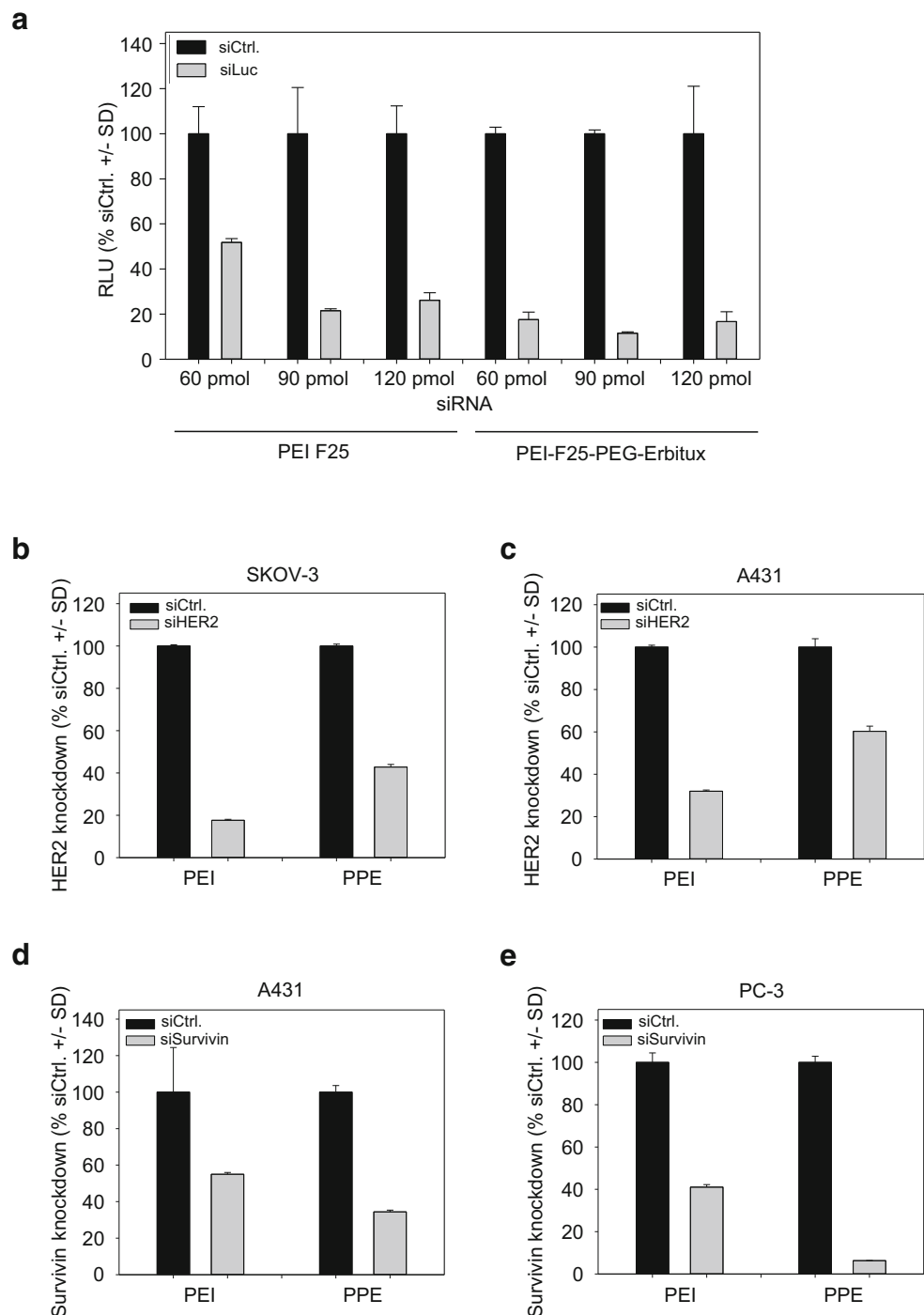
explored as indicated on the *x*-axis. (**b**, right): Uptake of P5Y-based complexes containing Alexa647-labeled siRNA (red), as determined by confocal microscopy; blue: cell nuclei (DAPI)

the P5Y/siRNA or P25Y/siRNA complexes required higher heparin concentrations for siRNA release than the corresponding complexes based on the parent PEI (Suppl. Fig. 1c). This was not seen in P2Y vs. 2 kDa PEI/siRNA complexes, indicating lower stability of the P2Y/siRNA complexes.

The degree of knockdown efficacies in stably luciferase-expressing SKOV-3-Luc cells was determined at different PEI/siRNA mass ratios as indicated in Fig. 2. All PEIs strongly benefitted from the tyrosine modification by a

marked enhancement of knockdown efficacies and the possibility to even switch to lower mass ratios as compared to the parent PEIs. Since excessive polymer may be associated with decreased biocompatibility, this is also beneficial with regard to reduced toxicity. Very low cytotoxicity was also confirmed in LDH release assays, demonstrating all values, except for 5 kDa PEI at the highest mass ratio, to be below the 10 % threshold of cytotoxicity [56] (Suppl. Fig. 1d).

**Fig. 3** Knockdown efficacies of PEG-PEI-Erbitux/siRNA complexes (PPE) for targeted siRNA delivery as compared to their non-functionalized PEI/siRNA counterparts. **a** Luciferase knockdown in stably luciferase-expressing SKOV-3-Luc cells; knockdown of the oncogenic receptor HER2 in **b** SKOV-3 and **c** A431 epidermoid carcinoma cells; knockdown of the oncogenic survival factor survivin in **d** A431 and **e** PC-3 prostate carcinoma cells. The knockdown experiments were performed in 6-well plates with 200 pmol siRNA per well



In the case of 10 or 25 kDa PEI, the tyrosine modification increased biological efficacies (Fig. 2c, d), with a maximum knockdown of almost 90 % (P10Y, mass ratio 3.75; Fig. 2c). The very low molecular weight (2 kDa) PEI was essentially inactive without tyrosine coupling, but reached a ~50–60 % knockdown upon its modification even at lower mass ratios (Fig. 2a). In the case of 5 kDa PEI, knockdown efficacy could only be reached with a comparably high mass ratio 20, while upon tyrosine modification, a >80 % knockdown was observed even at ratios as low as 2.5 (Fig. 2b). Biological activity was accompanied by profound siRNA uptake, as determined by confocal microscopy using Alexa647-labeled siRNA (Fig. 2b, right).

We thus conclude that the tyrosine modification strongly enhances biological efficacies, which also allows using low molecular weight PEIs that are particularly biocompatible.

### Covalent coupling of antibodies for the targeted delivery of complexes

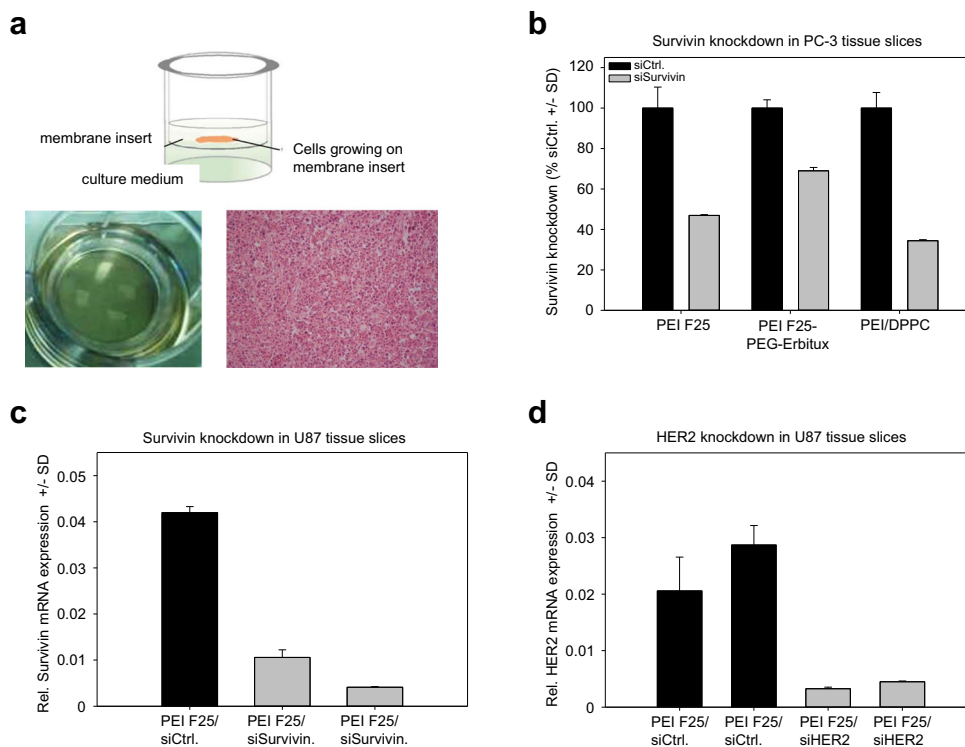
The concept of coupling ligands to nanoparticles as targeting moiety for their binding and selective uptake into target cells (see, e.g. [42, 57]) may provide a promising strategy for increasing biological efficacies and reducing off-target effects. Previously, we found that this first of all requires the shielding of PEI complexes in order to reduce their non-specific uptake mediated by the strongly positive zeta potential. The grafting of PEG provides an efficient tool for decreasing surface

charge and inhibiting non-specific interactions with non-target cells or other components of biological media [50, 58].

In our studies, we coupled the EGFR-specific monoclonal antibody cetuximab (Erbix®), which has been rather extensively explored as targeting moiety for various nanoparticles (see, e.g. [59–61]), to PEI via a heterobifunctional PEG linker as described previously [43]. Additionally, PEI was further PEGylated using monofunctional PEG for surface charge shielding. Optimal mass ratios for complex formation were determined (not shown). While complex sizes were slightly reduced upon PEG grafting and antibody coupling, this led to a marked decrease in the zeta potential (Suppl. Table 1). When transfecting stably luciferase-expressing SKOV-3-Luc cells, which display endogenously high EGFR levels on their surface, increased knockdown efficacies were observed in the case of PEG-PEI-Erbix as compared to the parent PEI F25-LMW (Fig. 3a). This was particularly true for low siRNA concentrations (see 60-pmol datasets).

We then switched to the knockdown of endogenously expressed target genes with relevance in tumor biology, i.e., the oncogenic receptor HER2 [62] or the oncogenic survival factor survivin [63]. In contrast to the luciferase data, the HER2 knockdown was less profound in the case of the complexes based on the PEG-PEI-Erbix conjugate as compared to PEI/siRNA complexes. This was observed in SKOV-3 cells (Fig. 3b) as well as in the epidermoid carcinoma cell line A431 (Fig. 3c), thus also confirming that this finding was not cell type-specific. In the same cell line, however, the knockdown of survivin was enhanced over PEI/siRNA

**Fig. 4** Tissue slice cultures as ex vivo model. **a** Schematic illustration of slice cultivation (*upper panel*), view onto tissue slices in culture (*lower left*), and a microscopic picture of a hematoxylin/eosin-stained section taken from a tissue slice for histology analysis (*lower right*). Figure modified from (Merz et al., pending revisions). Knockdown of endogenous target genes survivin (**b, c**) and HER2 (**d**) in PC-3 tumor xenograft tissue slices, comparing targeted PEG-PEI-Erbix/siRNA complexes and non-targeted PEI/siRNA complexes or DPPC/PEI/siRNA lipopolyplexes (**b**), and in U87 glioblastoma xenograft tissue slices using PEI/siRNA complexes (**c, d**)





complexes when employing the PEG-PEI-Erbtux/siRNA complexes for targeted siRNA delivery (Fig. 3d). This was even more so in PC-3 prostate carcinoma cells, leading to a very profound >90 % reduction of survivin mRNA levels (Fig. 3e).

Taken together, this demonstrates that targeted delivery may, at least under certain circumstances, indeed enhance biological efficacy, but that these effects may also depend on the protein used for targeted delivery and the target gene for knockdown. In fact, the discrepancies between the HER2 and the survivin knockdown data may well be explained by autocrine adaptation processes of the cells upon binding of Erbitux, as observed previously, e.g. after EGFR inhibition in glioma cells [64], thus also highlighting the necessity to determine optimal targets for delivery and knockdown.

### Tissue slice cultures as an ex vivo tumor model

While most in vitro studies rely on cultivated cells growing adherently on a plastic surface, these systems suffer from certain shortcomings, like the absence of the natural in vivo environment of the tumor cell, including tumor stroma. The ex vivo cultivation of intact tumor tissue provides a more relevant test system for therapeutic effects, avoiding the necessity of extensive in vivo studies. We have previously established tumor tissue slice cultures as ex vivo model [45, 46], allowing for the cultivation of intact tumor tissue for at least up to 14 days. The cultivation of the tissue slices at a liquid-air interface (Fig. 4a) also offers the possibility of directly accessing the tissue, without the need of the blood circulation for the delivery of drugs. We have extended this system towards the use of nanoparticles for the knockdown of a selected target gene (Merz et al., pending revisions). In the most recent studies presented here, we further explored tumor tissue from tumor xenografts generated in mice and subsequently explanted for slicing. A >50 % knockdown of survivin in PC-3 prostate carcinoma xenograft slices was observed upon their treatment with PEI/siRNA complexes or DPPC/PEI/siRNA lipopolyplexes (Fig. 4b). This also reflects that nanoparticles are able to penetrate into the tissue, since otherwise, an only less profound overall knockdown would be observed. When employing the above described PEG-PEI-Erbtux/siRNA complexes for targeted siRNA delivery, however, a somewhat less efficient ~35 % knockdown was observed. This may be readily explained by the higher binding affinity of the targeted nanoparticles towards their target cells, likely inhibiting penetration into deeper tissue areas due to their binding to the first cell layers reached. Thus, this also indicates that despite increased target cell specificity, it will have to be carefully considered under which circumstances a ligand-mediated targeted delivery really provides an advantage over less specific nanoparticle systems. In line with this, very profound knockdown efficacies were also observed in

tissue slices generated from U87 glioblastoma xenografts. This was true for survivin (Fig. 4c) as well as for HER2 knockdown (Fig. 4d). As to be expected, when comparing different siRNAs, differences in knockdown efficacies were found, thus also highlighting the need for identifying optimal siRNA sequences with regard to efficacy and absence of off-target effects.

Taken together, we demonstrate that the tissue slice culture model provides an attractive platform for testing and analyzing the nanoparticle (PEI/siRNA)-mediated knockdown of target genes of interest in a setting that resembles the in vivo situation more closely than tissue culture.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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