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RESEARCH PAPER

Poly(I:C)-Mediated Tumor Growth Suppression in EGF-Receptor Overexpressing Tumors Using EGF-Polyethylene Glycol-Linear Polyethylenimine as Carrier

David Schaffert • Melinda Kiss • Wolfgang Rödl • Alexei Shir • Alexander Levitzki • Manfred Ogris • Ernst Wagner

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ABSTRACT

Purpose To develop a novel polyethylenimine (PEI)-based polymeric carrier for tumor-targeted delivery of cytotoxic double-stranded RNA polyinosinic:polycytidylic acid, poly(I:C). The novel carrier should be chemically less complex but at least as effective as a previously developed tetra-conjugate containing epidermal growth factor (EGF) as targeting ligand, polyethylene glycol (PEG) as shielding spacer, 25 kDa branched PEI as RNA binding and endosomal buffering agent, and melittin as endosomal escape agent.

Methods Novel conjugates were designed employing a simplified synthetic strategy based on 22 kDa linear polyethylenimine (LPEI), PEG spacers, and recombinant EGF. The efficacy of various conjugates (different PEG spacers, with and without targeting EGF) in poly(I:C)-mediated cell killing was evaluated *in vitro* using two human U87MG glioma cell lines. The most effective polyplex was tested for *in vivo* activity in A431 tumor xenografts.

Results Targeting conjugate LPEI-PEG2 kDa-EGF was found as most effective in poly(I:C)-triggered killing of tumor cells *in vitro*. The efficacy correlated with glioma cell EGFR levels. Repeated intravenous administration of poly(I:C) polypexes strongly retarded growth of A431 human tumor xenograft in mice.

Conclusions The optimized LPEI-PEG2 kDa-EGF conjugate displays reduced chemical complexity and efficient poly(I:C)-mediated killing of EGFR overexpressing tumors *in vitro* and *in vivo*.

KEY WORDS epidermal growth factor · polyplex · receptor-mediated delivery · RNA delivery · tumor targeting

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D. Schaffert (✉) • M. Kiss • W. Rödl • M. Ogris • E. Wagner (✉)
Pharmaceutical Biotechnology, Department of Pharmacy
Center for Drug Research, Ludwig-Maximilians-Universität Munich
Butenandtstr. 5-13
81377 Munich, Germany
e-mail: david.schaffert@cup.uni-muenchen.de
e-mail: ernst.wagner@cup.uni-muenchen.de

D. Schaffert • M. Kiss • W. Rödl • M. Ogris • E. Wagner
Center for NanoScience (CeNS)
Ludwig-Maximilians-Universität Munich
Munich, Germany

A. Shir • A. Levitzki
Unit of Cellular Signaling, Department of Biological Chemistry
The Hebrew University of Jerusalem
Jerusalem, Israel

ABBREVIATIONS

HBG	Hepes buffered glucose (5% (w/v) glucose, 20 mM Hepes, pH 7.4)
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid
IEX	ion-exchange chromatography
LPEI	linear polyethylenimine with an average molecular weight of 22 kDa
OPSS	orthopyridyl dithio
PEG	polyethylene glycole
p(I)	poly inosinic acid
poly(Glu)	polyglutamic acid
poly(I:C)	poly inosinic-cytidylic acid
SEC	size exclusion chromatography

INTRODUCTION

The rapid progress in cancer therapy over the last few years has led to the identification of new therapeutic targets, the introduction of new therapeutic technologies like antibody or siRNA treatment and the implementation of personalized

treatment regimes in therapy. But despite the effort invested in these new approaches, classical chemotherapeutic agents such as doxorubicin or cisplatin are still widely employed in clinics. The inherent drawbacks of these therapeutic approaches are the sometimes severe side effects and the intrinsic or acquired resistance of cancer cells towards the therapeutic drug. In an ideal therapeutic regime the cancer cells are effectively destroyed by the drug without harming the surrounding cells. This can be achieved by drug-targeting or by targeting cancer-specific cellular pathways.

Hence, new, promising nucleic acid-based therapeutic concepts like antisense therapy or the application of siRNA have moved into the focus of scientific interest. But the application of gene or oligonucleotide-based approaches has its own pitfalls, namely the identification of therapeutic target proteins and the specific delivery of the appropriate nucleic acid into the cancer cells. The delivery is especially challenging, as nucleic acids need lipid-based or polymer-based carrier systems (1–8) which protect them in the extracellular environment, effectively transport them to the effector site and facilitate their release into the cytosol.

To circumvent acquired chemoresistance of tumor cells and to increase the therapeutic efficacy, a triple effector strategy has been developed, combining targeted delivery, apoptosis induction and the immunostimulatory properties of the artificial dsRNA poly(I:C) (9). By targeting the EGF receptor which is overexpressed in a variety of tumors, a better uptake of poly(I:C) into target cells is possible, followed by interferon induction and apoptosis. Intratumoral application of poly(I:C)/cationic polymer complexes (polyplexes) in an orthotopic glioblastoma model or two other EGF receptor overexpressing tumor models caused complete tumor regression in nude mice (9). These very promising therapeutic results were based on poly(I:C) polyplexes with a tetra-component conjugate, consisting of 25 kDa branched polyethylenimine (brPEI), EGF as targeting ligand and polyethylene glycol (PEG) for shielding (10,11), and a synthetic derivative of the lytic peptide melittin (12,13). The latter was found to be strictly required for cytosolic delivery of poly(I:C) and therapeutic efficacy. Though effective, the tetraconjugate is not practical for further development due to its complexity. In the current communication, we report the synthesis of an improved EGF/PEI-based carrier with reduced complexity. The chemically poorly defined brPEI was replaced by the analogous linear 22 kDa polymer (LPEI). LPEI can be synthesized in GMP-compatible form (14), has already been tested in human clinical trials as DNA formulation, and was found to be more effective as brPEI in several applications (15–17). The generation of a melittin-free conjugate was possible by selecting an optimized PEG/PEI ratio (equimolar amounts using 2 kDa PEG). The newly developed LPEI-based poly(I:C)-carrier system exhibits the key features of the old tetraconjugate, namely EGF receptor targeting and effective payload release into the cytosol of tumor cells. The new conjugate shows an improved therapeutic efficiency combined with a simpler synthesis route, allowing the convenient synthesis of larger amounts of the carrier.

MATERIALS AND METHODS

Materials

Dimethylsulfoxide purissimum (DMSO) and ethanol absolutum (EtOH) were obtained from Sigma-Aldrich GmbH (Deisenhofen, Germany). Water was used as purified, deionized water. Poly(2-ethyl-2-oxazoline) 50 kDa was obtained from Sigma-Aldrich. ω-2-Pyridyldithio polyethylene glycol α-succinimidylesters (NHS-PEG-OPSS) were synthesized by Rapp Polymere GmbH (Tübingen, Germany) and used without further purification. SPDP was synthesized as described in (18); murine recombinant epidermal growth factor (EGF) was obtained from Peprotech GmbH (Hamburg Germany). *all-D*-Melittin peptide with the sequence *H*-CIGAVLKVLTTGLPALISWIKRKRQQ-*OH* (12) was synthesized by IRIS Biotech. Sephadex G-25, Sephadex G-10 were obtained from GE Healthcare Europe GmbH (Freiburg, Germany). MacroPrep High S was provided by BioRad GmBH, München. Cell culture media, antibiotics, and fetal calf serum (FCS) were purchased from Life Technologies (Karlsruhe, Germany). Dialysis was performed with Spectra/Por membranes (molecular mass cut-off 10 kDa; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 4°C. Polyinosinic-cytidylic acid (poly(I:C)) and polyinosinic acid (poly(I)) were obtained from Sigma-Aldrich GmbH (Deisenhofen, Germany).

Synthesis of LPEI 22 kDa (Free Base Form)

Synthesis of LPEI was performed analogous to published procedures (17,19) with modifications. Poly(2-ethyl-2-oxazoline) (5 g) were suspended in 50 ml of 30% hydrochloric acid. The mixture was refluxed for 48 h, yielding a fine white precipitate. The solid was isolated by filtration and washed four times using 30% HCl to remove traces of propionic acid. The resulting LPEI hydrochloride was air-dried overnight, dissolved in 200 ml distilled water and freeze-dried. Yield: 3.5 g, 85% (^{1}H-NMR, D_2O, 400 MHz: broad singlett 3.5 ppm) LPEI hydrochloride (2.5 g) were dissolved in 75 ml of 1 M NaOH at 100°C. The solution was cooled to room temperature, and the resulting LPEI precipitate isolated. The gel-like precipitate was washed 3 times with 75 ml 1 M NaOH and 5 times with 75 ml distilled water. The resulting viscous gel was transferred into a round-bottom flask, shock frosted using

liquid nitrogen and lyophilized, yielding 1 g (76%) of a white, fluffy lyophilizate.

Removal of Low Mw Impurities from LPEI and brPEI

Fifty mg of LPEI (hydrochloride) or brPEI (free base) were dissolved in 1 ml water, and the pH was adjusted to 7.0 using NaOH or HCl, respectively. Small molecular weight fractions of the polymer were removed by SEC chromatography using a G-25 preparative grade Sephadex column and a 20 mM HEPES (pH 7.4) buffer for elution. The PEI-containing fractions were pooled and concentrated. PEI concentrations were determined using photometric copper assay (20) for LPEI, or TNBS (2,4,6-trinitrobenzene sulfonic acid) assay (21) for brPEI, respectively.

Synthesis of EGF-SH

Mercapto-modified EGF was synthesized analogously as described in (10). A solution of 10 mg of EGF (1.65 μmol, recombinant, murine) in 1.0 ml of 20 mM HEPES buffer pH 7.1 was mixed with a solution of SPDP (5.2 mg, 16.5 μmol) in 0.5 ml EtOH, resulting in a final concentration of 30% EtOH. After 2 h reaction time the resulting EGF-PDP was purified by SEC using a Sephadex G-25 superfine column and pH 7.1 HEPES/30% EtOH buffer for elution. The product-containing fractions were collected and concentrated in a speedvac. Five mg of the resulting EGF-PDP in 2.5 ml of 20 mM HEPES pH 7.1 were treated with a 50-fold molar excess of DTT for 15 min under argon atmosphere. EGF-SH was purified by SEC on a Sephadex G-10 column using 20 mM HEPES pH 7.1 for elution yielding 3.5 mg EGF-SH (determined by A_{280}).

Synthesis of LPEI-PEG-OPSS Conjugates

LPEI (20 mg free base form, 0.9 μmol) was dissolved in 1 ml of EtOH by shaking for 30 min at 30°C. The use of ethanol as solvent was superior over aqueous buffers and various other organic solvents in terms of reproducibility and yield. After complete dissolution, 2.25 μmol of the appropriate (2 kDa, 5 kDa or 10 kDa) NHS-PEG-OPSS were added, and the resulting mixture was agitated for 3 h. The resulting conjugate was purified using ion-exchange chromatography (20 mM HEPES pH 7.1, elution with 20 mM HEPES containing 3 M NaCl) followed by dialysis against 20 mM HEPES pH 7.1. Substitution grade was calculated by A_{343} after DTT-induced 2-pyridinethione release and/or ^{1}H-NMR analysis.

Synthesis of LPEI-PEG-EGF Conjugates

LPEI-PEG-OPSS (5 mg, corresponding to 0.2 μmol of OPSS) in 20 mM HEPES pH 7.1 were mixed with a 1.5 molar surplus of EGF-SH and incubated until A_{343} indicated complete turnover. The resulting conjugate was purified by SEC on a Sephadex G-25 column using 20 mM HEPES pH 7.1 as eluent and concentrated using a speedvac. Concentration was determined by photometric copper assay. According to this procedure, LPEI-PEG2 kDa-EGF, LPEI-PEG5 kDa-EGF and LPEI-PEG10 kDa-EGF conjugates were obtained with a molar ratio of 1 : 0.9 : 0.9, 1 : 1.2 : 1.2, or 1 : 1.4 : 1.4, respectively.

Synthesis of brPEI Tetraconjugate (Mel-brPEI-PEG-EGF)

EGF-PEG-brPEI-Mel was synthesized like described before (9). Briefly, EGF-SH is anchored to NHS-$PEG_{3.4k}$-maleinimide, the resulting EGF-$PEG_{3.4k}$-NHS-Linker is conjugated to brPEI and the resulting conjugate purified by SEC. The construct is subsequently modified with SPDP, purified by SEC and in the last step modified with melittin-SH, followed by SEC purification. This resulted in a conjugate with the nominal composition of EGF: PEG: brPEI: Mel= 2.5: 2.5: 1: 5.

Formation of Polyplexes

Polycation/RNA polyplexes were prepared as described (12). Briefly, indicated amounts of RNA poly(I:C) or poly(I) and polycation were diluted in a similar volume of HBG pH 7.1 and mixed rapidly by pipetting. Polyplexes were incubated at room temperature for 20 min prior to use. All tested formulations were mixed at a molar PEI nitrogen-to-RNA phosphate (N/P) ratio of 6, corresponding to a PEI/RNA weight/weight ratio of 0.8.

Size and Zetapotential Measurement

The size of PEI/poly(I:C) complexes was determined by dynamic light scattering. Polyplexes were prepared as described for the delivery experiments and measured using a zetasizer nano (Malvern Instruments, Herrenberg, Germany). For zeta-potential measurement the polyplexes were diluted using 1 mM NaCl to yield an end concentration of 10 μg poly(I:C)/ml. Dispersion Technology Software 5.0 was used for data acquisition and analysis.

Cell Culture and Cell Killing Assay *In Vitro*

U87MG and U87MGwtEGFR human glioblastoma cells were cultured on collagen-coated flasks in DMEM (1 g of glucose/L) supplemented with 10% fetal calf serum (v/v) and 1% penicillin/streptomycin (v/v). U87MGwtEGFR were maintained under G418 selection pressure. Always two parallel polyplex series were carried out in separate 96-well

plates (TPP, Transadingen, Switzerland), one for the determination of cell killing efficacy of poly(I:C) polyplex formulations and one for the determination of cytotoxicity using analogous polyplexes of poly(I) as control. Cells were seeded 24 h prior to transfection with a density of 1×10^4 cells in 200 µl of culture medium per well. Immediately before transfection, medium was removed, and 100 µl of a dilution of transfection complexes in serum-containing culture medium were added to the cells. After 4 h of incubation at 37°C, polyplex-containing medium was replaced by 200 µl of fresh serum-containing medium. All experiments were performed in triplicates. Cell killing was evaluated 48 h after treatment by methylthiazole tetrazolium (MTT)/thiazolyl blue assay as described in (22). Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a micro plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria). Metabolic activity was expressed relative to the metabolic activity of untreated control cells, defined as 100%.

A431 cells were cultured on collagen-coated flasks in DMEM (1 g of glucose/L) supplemented with 10% fetal calf serum (v/v) and 1% penicillin/streptomycin (v/v). Two parallel polyplex series were carried out, one for the determination of cell killing efficacy of poly(I:C) polyplex formulations and one for the determination of cytotoxicity using analogous polyplexes of polyglutamate (poly(Glu)) as control. Cells were seeded 24 h prior to transfection with a density of 4×10^3 cells in 200 µl of culture medium per well. Immediately before transfection, medium was removed, and 100 µl of a dilution of transfection complexes in serum-containing culture medium were added to the cells. After 4 h of incubation at 37°C, 100 µl of fresh serum-containing medium were added. All experiments were performed in duplicates. Cell killing was evaluated by methylthiazole tetrazolium (MTT)/thiazolyl blue assay as described above.

In Vivo Study

In vivo anti-tumor activity of EGFR-targeted poly(I:C) PEI polyplexes was measured using subcutaneous A431 mouse xenografts. Before the experiment, human epidermoid carcinoma A431 cells were cultured in DMEM supplemented with 10% fetal calf serum (v/v) and 1% penicillin/streptomycin. Two-million A431 cells were dissolved in 200 µl of phosphate-buffered saline and injected subcutaneously into the right flank of immune compromised female athymic nude mice (Nude-Hsd, 5 weeks old). Volume of the growing tumors was calculated as follows: $V=LW^2/2$ (L = length, W = width). When the tumors reached average volume of 100 mm^3, mice were randomly divided into five groups (five mice per group), and treatment was initiated. The complexes were delivered by intravenous injection every 48 h for 2 weeks. The first group received poly(I:C)/Melittin-PEI25-PEG-EGF (Poly IC/MPPE) polyplexes in HBG buffer at 0.1 µg poly(I:C)/µl buffer (the total dose of poly(I:C) was 10 µg/injection). The second group received poly(I:C)/LPEI-PEG-EGF (poly(I:C)/PPE) polyplexes in HBG at the same dose and concentration. The control groups (poly(Glu)/MPPE and poly(Glu)/PPE) were treated with the same doses of polymer conjugates but replacing poly(I:C) by polyglutamate poly(Glu). The fifth group did not receive any treatment. Tumor volume was measured twice a week until day 14.

Statistical Analysis

Results were expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for evaluating statistical significance. Statistical analysis was performed with GraphPad Prism 4.0. Statistical significance was set when $P<0.05$.

RESULTS

Synthesis of LPEI-PEG Conjugates

A comparison of the chemical syntheses of the LPEI triconjugate and the brPEI tetraconjugate is presented in Fig. 1. LPEI-PEG-EGF conjugates were synthesized by a two-step procedure. In the first step, NHS-PEG-OPSS is anchored to LPEI via its amine-reactive NHS function using EtOH as solvent. The resulting PEGylated carrier can now be modified using any thiol-containing ligand using an orthogonal disulfide exchange reaction which can be spectroscopically monitored.

The brPEI tetraconjugate (EGF-PEG-brPEI-Mel) synthesis consists of four consecutive reaction steps with intermediate purification. In the first step, EGF-SH is conjugated to NHS-PEG3.4 kDa-maleinimide, which is subsequently grafted to brPEI. This conjugate is subsequently modified with SPDP, purified and, in the last step, modified with melittin-SH.

Polyplex Formation and Biophysical Characterization

The biophysical characteristics of the different conjugates were determined by zeta potential and particle size analysis (Table 1). Polyplexes prepared by complexation of unmodified PEIs result in well-compacted particles (size ~ 120 nm) characterized by relatively high zeta potentials of ≥ +30 mV. The introduction of a PEG-shielding domain leads to significant drop of zeta potential. This was more pronounced for the modification with 10 kDa PEG than for 2 kDa PEG (+11 *vs* +24 mV) or 5 kDa PEG (+14 mV). The attachment of the EGF leads to a slight increase in

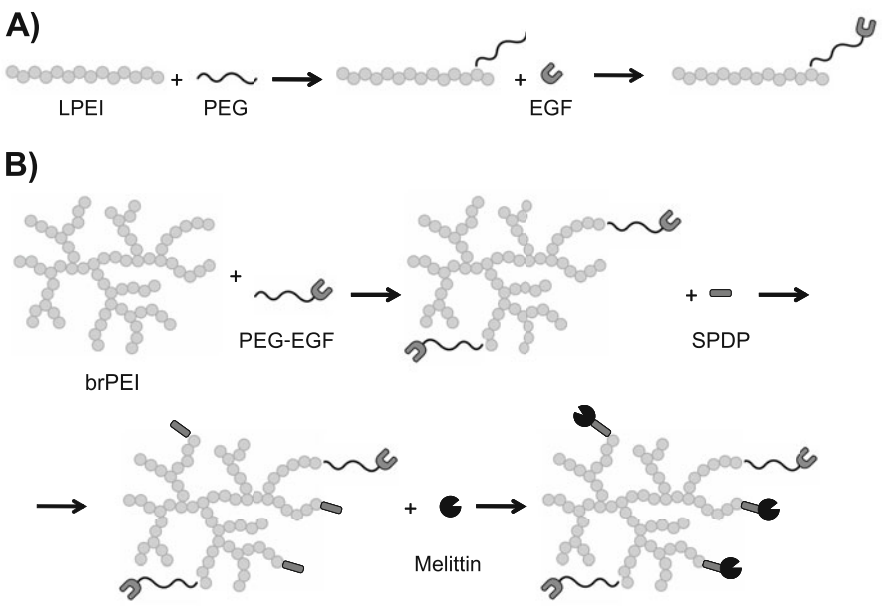

Fig. 1 Conjugate syntheses. **A** LPEI-PEG-EGF triconjugates **B** Mel-brPEI-PEG-EGF tetraconjugate.

zeta potential for polyplexes with all PEGylated PEIs (+17 mV *vs* +27 mV *vs* +20 mV).

The brPEI tetraconjugate shows a very low zeta potential (+4 mV) and a larger less uniform diameter (233±122 nm), most probably resulting from the massive modifications of the brPEI backbone (Table 1). The results are also consistent with the fact that the brPEI conjugate was modified with approximately 2.5 M equivalents of PEG3.4 kDa-EGF chains, as opposed to about one PEG-EGF for the other three conjugates.

The poly(I:C) binding capabilities of the conjugates were comparable as determined in an agarose gel shift assay (Supplemental Material Figure S1 and data not shown). A heparin displacement assay (Supplemental Material Figure S2) revealed small differences between the different polymers. Most significant, a slightly weaker binding of poly(I:C) was found with LPEI (and LPEI conjugates) as opposed to branched brPEI (and the brPEI tetraconjugate). This might have a positive impact on poly(I:C) delivery and intracellular release (see below, next section). PEGylation of LPEI with 2 kDa PEG did not alter poly(I:C) binding, but modification with 10 kDa PEG further weakened poly(I:C) binding. The effect of PEGylation, however, was far less pronounced than the influence of the cationic polymer carrier (LPEI *vs.* brPEI).

Table 1 Biophysical Characterization

Conjugate	Zeta potential [mV]	Size [nm]
brPEI	30.0±1.6	120.1±1.1
LPEI	31.8±0.8	122.9±2.1
LPEI-PEG10K	11.1±0.5	114±3.0
LPEI-PEG5K	14.2±1.3	137.8±2.1
LPEI-PEG2K	23.9±2.7	121.6±1.9
LPEI-PEG10K-EGF	16.9±1.4	143.9±33.4
LPEI-PEG5K-EGF	19.5±0.6	210.5±2.9
LPEI-PEG2K-EGF	27.3±2.3	210.4±4.1
Mel-brPEI-PEG3.4K-EGF	3.5±0.9	233.0±122.0

In Vitro Anti-tumoral Activity of Poly(I:C) Polyplexes

Poly(I:C) delivery properties were determined by a cytotoxicity assay using the p(I:C)-sensitive EGF-R-overexpressing glioblastoma cell line U87MGwtEGFR. To differentiate between poly(I:C)-induced cell death and a potential carrier toxicity, single-stranded poly(I) was used as control, as it is reported that the single-strand RNA does not induce apoptosis (23,24).

Plain PEI/Poly(I:C) Polyplexes

To evaluate the suitability of LPEI as a better-defined carrier backbone in poly(I:C) delivery, plain LPEI was compared to brPEI without any further modification of the polymers. In the tested poly(I:C) concentration range of 0.25–2.5 μg/ml, brPEI/poly(I:C) polyplexes showed no effect on the viability of U87MGwtEGFR cells (Fig. 2 top left panel). LPEI demonstrates a superior delivery efficiency at concentrations as low as 0.25 μg/ml, but this is accompanied by a fast shift into unspecific cytotoxicity beginning at 1 μg/ml (Fig. 2 top right panel). For DNA transfections, a concentration of 0.8 μg/ml is routinely well tolerated with only moderate toxicity, indicating a LPEI-independent toxicity mechanism. This effect severely limits the use of unmodified LPEI because of unspecific uptake and a small therapeutic window.

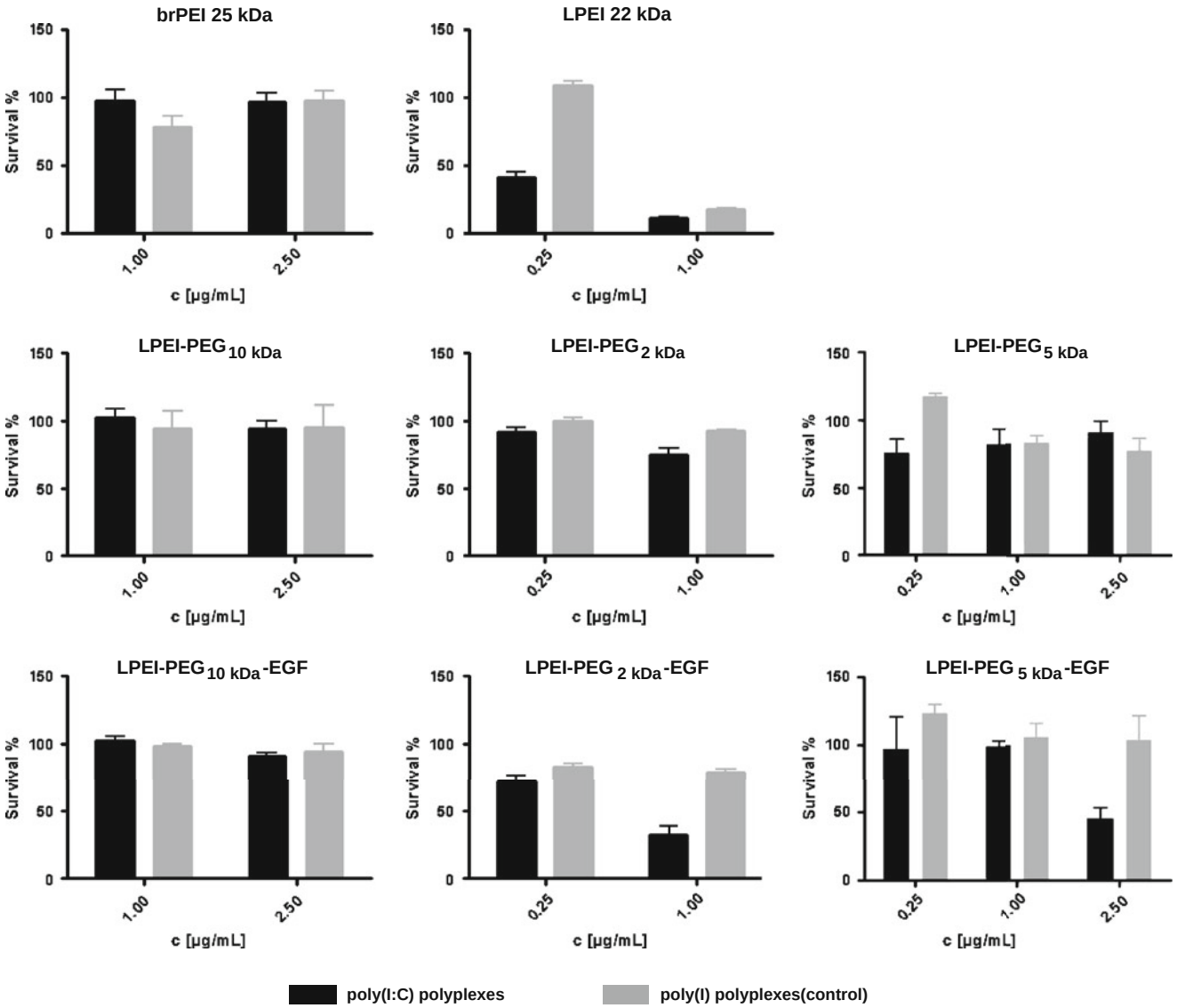

Fig. 2 *In vitro* anti-tumoral activity of poly(I:C) polyplexes against U87MGwtEGFR glioma cells. Comparison of brPEI *versus* LPEI (*top panels*), LPEI-PEG10 kDa *versus* LPEI-PEG2 kDa and PEG5 kDa (*middle panels*), and receptor-targeted conjugates LPEI-PEG10 kDa-EGF *versus* LPEI-PEG2 kDa-EGF and LPEI-PEG5 kDa-EGF (*lower panels*). Please note the different dosages for ineffective conjugates and effective conjugates. For each dosage the same dose of poly(I) polyplexes served as negative control.

The extent of the far higher potency of LPEI compared to brPEI is surprising, but consistent with previous findings for DNA delivery, for example (16,25). A better reversibility of nucleic acid complexation (see also section above) is hypothesized as key issue. Itaka *et al.* (25) reported on an enhanced intracellular disassembly of LPEI as compared to brPEI DNA polyplexes by intracellular FRET experiments. Intracellular cytosolic release appears to be a critical requirement also for accessibility of polyIC.

PEGylated PEI/Poly(I:C) Polyplexes

Various groups described the beneficial role of PEGylation on cytotoxicity of PEI polyplexes, but the grafting of PEG chains onto PEI may also be accompanied by a drop in delivery performance. This has been demonstrated for plasmid DNA delivery (26–31); the situation may, however, be different in the case of siRNA delivery (32). Therefore, to evaluate the optimal PEG molecular weight for poly(I:C) delivery, PEG10 kDa, PEG5 kDa and PEG2 kDa were grafted onto the brPEI or LPEI backbone. Figure 2 middle panels show a huge impact of PEGylation of LPEI on the delivery efficiency. The attachment of a single PEG10 kDa chain to LPEI renders the conjugate inactive. At concentration of 2.5 µg/ml of poly(I:C) or even higher (5 µg/ml, Supplemental Material Figure S3), no significant cell killing is observed. Modification with PEG2 kDa or PEG5 kDa reduces the efficacy and cytotoxicity profile of the

corresponding poly(I:C) polyplexes (Fig. 2 middle panel) considerably.

Targeted EGF-Containing PEGylated PEI/Poly(I:C) Polyplexes

To evaluate the influence of a targeting ligand onto the delivery efficiency of the LPEI-PEG conjugates, murine EGF was attached at the distal end of the 2 kDa, 5 kDa or 10 kDa PEG spacer. The conjugates were tested as poly(I:C) polyplexes for their cell killing activity (Fig. 2 bottom panel). As expected, introduction of the targeting ligand improved the activity of the PEG2 kDa-LPEI conjugate, leading to an increased activity at lower concentrations with more than 60% cell killing at 1 µg/ml (Fig. 2 bottom panel). Cell killing was even more pronounced at 2.5 µg/ml, but poly(I) control polyplexes also triggered some killing (Supplemental Material Figure S3). Interestingly, the incorporation of EGF into the PEG10 kDa-LPEI conjugate did not recover any significant cytotoxic activity of the LPEI-PEG10 kDa-EGF polyplexes (Fig. 2 bottom panel), even at a higher dose of 5 µg/ml (Supplemental Material Figure S3). The LPEI-PEG5 kDa-EGF conjugate mediated specific poly(I:C) cell killing, but only at the higher 2.5 µg/mL dose (Fig. 2 bottom panel, right).

Comparison of the brPEI Tetraconjugate with the LPEI Triconjugate

The two conjugates, tetraconjugate EGF-PEG-brPEI3.4 kDa-Mel and triconjugate LPEI-PEG2 kDa-EGF were compared by testing on U87MG (moderate levels of EGFR) and EGFR-over-expressing U87MGwtEGFR cells (receptor levels see Supplemental Material Figure S4). Figure 3a shows an only limited efficacy on U87MG cells, with marked activity only at higher concentrations of 2.5 µg/ml (data not shown). Testing EGFR over-expressing U87MGwtEGFR cells (Fig. 3b), bioactivity was more pronounced with the new triconjugate, resulting in a 60% reduction of cellular viability by treatment at a concentration of 1 µg/ml poly(I:C). On both cell lines, a 2.5-fold higher concentration of the old tetraconjugate had to be applied to obtain a similar cell killing effect. Effects of the poly(I:C) treatment on cell morphology at 48 h after treatment are shown in Fig. 4.

The two conjugates were also compared using the EGFR overexpressing epidermoid carcinoma cell line A431 (Fig. 5). In these and the following experiments, polyplexes of the nontoxic polyanion polyglutamic acid served as negative control. Efficient and poly(I:C)-specific cell killing was obtained at the lowest tested 1 µg/ml poly(I:C) dose in the case of the LPEI-PEG2 kDa-EGF conjugate, whereas high doses were required in the case of the tetraconjugate.

In Vivo Anti-tumor Activity

In vivo anti-tumor activity of EGFR-targeted poly(I:C)/PEI polyplexes was examined using nu/nu mice bearing subcutaneous A431 tumors. Conjugate delivery activity was determined by tumor volume analysis after systemic application of tetra- and triconjugate and the control formulations. Intravenous administration of 0.5 mg/kg poly(I:C) started at day 0 and was repeated every second day, for a total of seven times. Measurement of the average body mass of the mice showed that the mice tolerated the treatment well.

As shown in Fig. 6, the tumor volume of control/untreated groups was about 12 times larger then the average tumor volume at day 0, indicating rapid tumor growth and no tumor growth inhibition by the polyglutamate control polyplexes. Treatment with either EGF triconjugate or EGF tetraconjugate resulted in significantly decreased tumor growth speed and tumor end volume. After 14 days of treatment (seven injections) the mean tumor volume of the tetraconjugate group was four-fold increased, while treatment with the triconjugate led to an only doubled tumor volume. The anti-tumoral effect was only observed in the poly(I:C) groups, showing significantly

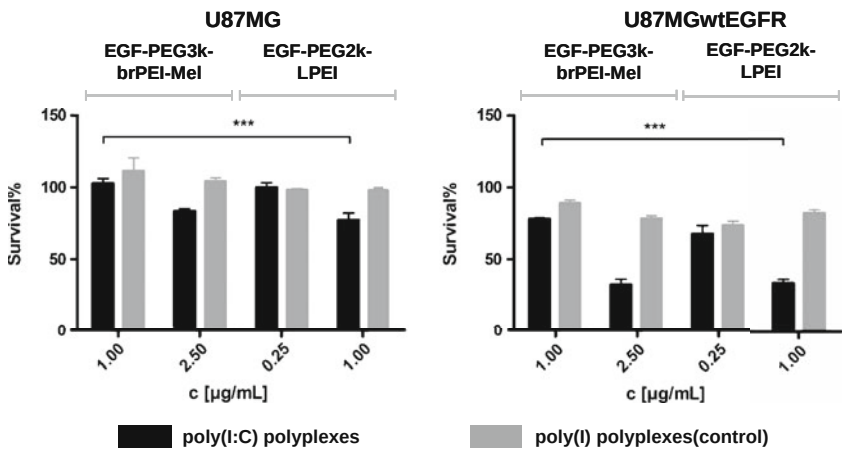

Fig. 3 *In vitro* anti-tumoral activity of poly(I:C) polyplexes. Comparison of the two EGF-conjugates (old tetraconjugate *versus* new triconjugate LPEI-PEG2 kDa-EGF) using U87MG glioma cells with low ('U87MG') or high ('U87MGwtEGFR') levels of EGF receptor. The same doses of poly (I) polyplexes served as negative controls.

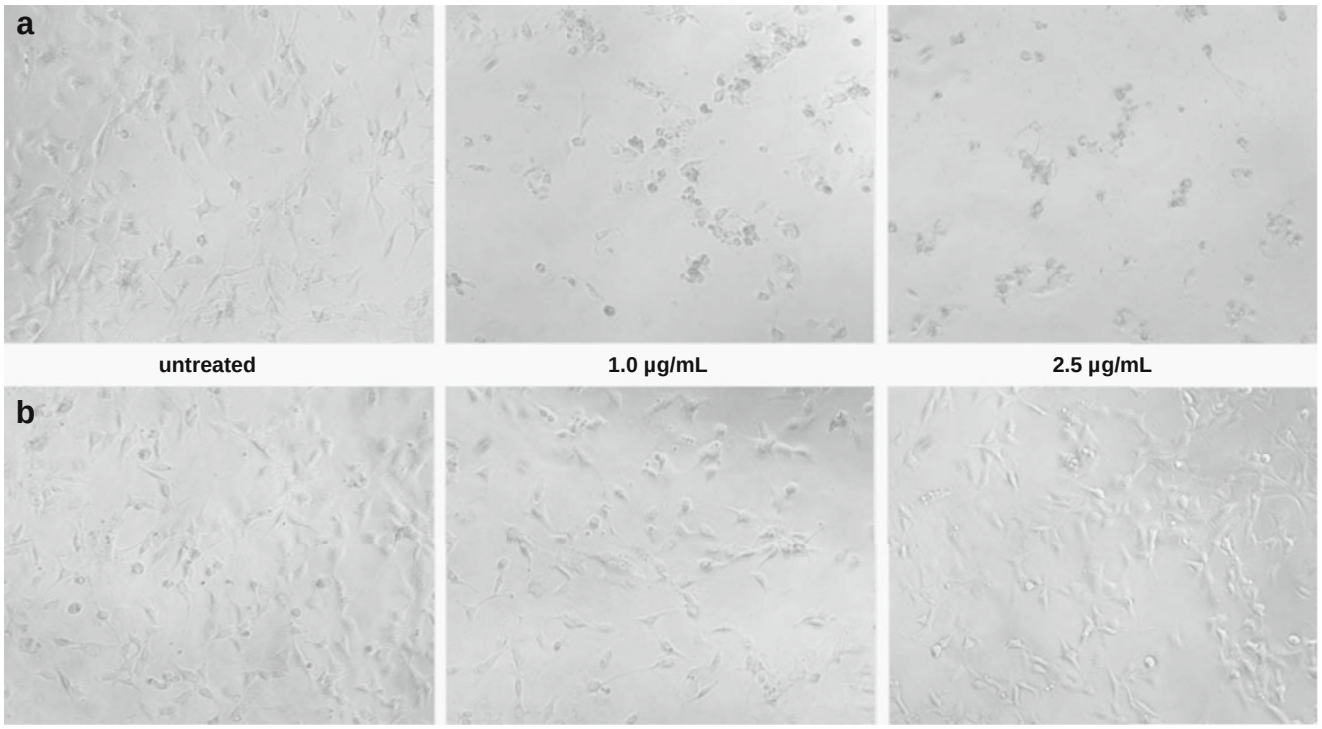

Fig. 4 Cell morphology of LPEI-PEG2 kDa-EGF polyplex treated U87MGwtEGFR cells at 48 h after transfection, **a** poly(I:C) treated cells; **b** poly(I) treated cells (control).

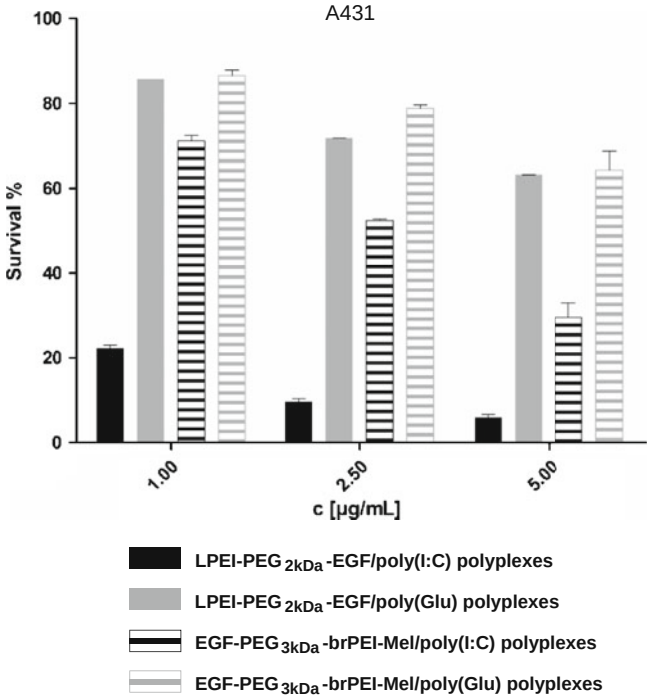

Fig. 5 *In vitro* anti-tumoral activity of poly(I:C) polyplexes against A431 cells. Comparison of the two EGF-conjugates (old tetraconjugate *versus* new triconjugate LPEI-PEG2 kDa-EGF). The same doses of poly(Glu) polyplexes served as negative controls.

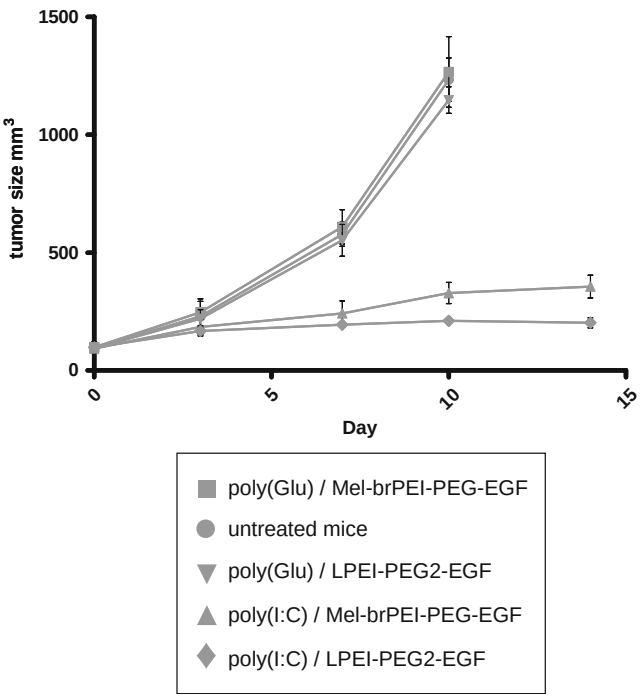

Fig. 6 *In vivo* anti-tumor activity of EGFR targeted poly(I:C) PEI polyplexes. *In vivo* anti-tumor activity of EGFR targeted poly(I:C) PEI polyplexes was measured using s.c. A431-bearing nude mice. Tumor volume analysis after i.v. injection of the two different formulations of poly (I:C) was done. Each group included 5 mice. Administration of 10 μg pI:C started on day 0 and was repeated on days 2, 4, 6, 8, 10, 12, for a total of 7 times. Tumor volume was measured twice a week until day 14.

decreased tumor growth progression compared to the control group.

DISCUSSION

A virally infected host organism reacts with multiple innate and acquired defense mechanisms to avoid further virus spread, including humoral and cellular immune responses against proteins and viral particles, and also responses against viral nucleic acid intermediates. A series of toll-like receptors (TLRs) at the cell surface and in endosomal vesicles and also cytosolic factors recognize viral nucleic acids which have different properties as compared to endogenous cellular RNA and DNA. This recognition often triggers inflammatory and interferon responses, shut-down of protein production and suicide of infected cells by apoptosis.

Synthetic viral nucleic acid analogs have been therapeutically applied as immunostimulatory and cytotoxic DNAs and RNAs (33–36). Poly(I:C) and analogs thereof (23,37–40) mimic double-stranded RNA of virus-infected cells which via endosomal toll-like receptor TLR3 and cytosolic helicase mda-5 stimulation activate different pro-apoptotic processes simultaneously. This makes poly(I:C) an interesting tool for cancer treatment because the differently triggered host-cell-killing mechanisms reduce the probability of developing acquired chemoresistence; they lead to cell death, and the additional expression of anti-proliferative interferons and other cytokines and chemokines inhibits growth of neighbouring cancer cells that have not been "infected" with poly(I:C) (9).

Untargeted poly(I:C) and analogs have already been applied as adjuvants in cancer-directed human immunotherapy studies, with some limited success but by far not all applications (38–40). For example, poly(I:C) stabilized by polylysine and carboxymethylcellulose applied intramuscularly three times a week for 4 weeks as a single agent therapy did not improve progression-free survival of anaplastic glioma patients in a phase II study (39). To fully exploit the therapeutic potential of both immune stimulation and tumor cell killing, poly(I:C) has to be delivered intracellularly into endosomes and also the cytosol, in a tumor-targeted fashion. Obviously, delivery presents the major bottleneck. Both liposomal and polymer-based strategies have been developed for poly(I:C). These include MHC antibody-targeted or pH-sensitive liposomes (41,42), lipoplexes (23,43), nontargeted polymer formulations (38) or, as outlined in our previous work, EGF receptor-targeted poly(I:C) polyplexes (9). In the latter paper, Shir *et al.* demonstrated killing of EGFR-overexpressing human tumors including glioblastoma upon local administration. Polyplexes consisted of poly(I:C) complexed with either one or two branched polyethylenimine (brPEI) conjugates comprising recombinant EGF as targeting ligand, PEG as shielding domain (10,11,44,45), and synthetic melittin peptide as endosomal release agent. Both targeting and endosomal domain were found to be essential for the observed biological activity (9).

The current work has been based on the task to develop an improved PEI-based carrier for poly(I:C) comprising all the mentioned EGFR targeting, PEG shielding, and endosomal release functions, but providing them within a polymer conjugate of reduced chemical complexity. This was achieved in the following way. First, the chemically poorly defined brPEI was replaced by the analogous linear 22 kDa polymer LPEI (11–13) which can be synthesized in GMP compatible form (14) and has already been tested in human clinical trials for DNA delivery. Due to its higher inherent potency over brPEI (16,25), plain LPEI actually mediated *in vitro* cell killing independent of receptor targeting or endosomolytic melittin (Fig. 2, upper panel). This effect, however, was accompanied by an unspecific, non-poly(I:C)-related cytotoxicity at higher polyplex doses, as demonstrated with poly(I) control polyplexes.

Second, LPEI was conjugated with PEG molecules of different molecular weight (2 kDa, 5 kDa and 10 kDa). PEGylation strongly reduced the cytotoxicity but also poly(I:C)-based cell killing. For 10 kDa PEG and 5 kDa PEG, the activity was lost; for 2 kDa PEG, only a hint of activity was obtained (Fig. 2, middle panel). Finally, incorporation of EGF as receptor-targeting ligand restored activity for the LPEI-PEG2 kDa conjugate but not LPEI-PEG10 kDa conjugate (Fig. 2, lower panels). The LPEI-PEG5 kDa mediated moderate activity at a higher dose. Such a "PEG dilemma" (indirect correlation of shielding and efficacy) is consistent with many previous observations by several laboratories (26,28,46–48) and might be explained by the fact that for endosomal membrane disruption stable PEG-shielding is counter-productive (30,49–51). Apparently the window between shielding/targeting specificity and efficient intracellular delivery is narrow.

The newly developed LPEI-PEG2 kDa-EGF conjugate exhibits the key features of the old tetraconjugate, namely higher potency on EGFR-overexpressing U87MGwtEGFR gliomas as compared to low-expressing U87MG cells (Fig. 3). An approximately 2.5-fold improved therapeutic efficiency was observed *in vitro* in comparison to the old conjugate on U87MGwtEGFR gliomas (Fig. 3). An efficient and specific poly(I:C)-mediated cell killing was also obtained with A431 cells (Fig. 5). These epidermoid carcinoma cells express particularly high levels of EGF receptor. EGFR density is described with 2×10^6/cell, higher than U87MGwtEGFR (1×10^6/cell) and much higher than U87MG (1×10^5/cell) (52,53). Most encouraging, systemic intravenous administration of poly(I:C) polyplexes were able to strongly retard growth of distant subcutaneous A431

tumors *in vivo*. The treatment was well tolerated by the mice. Once again, the effect was dependent on poly(I:C) as key component of the formulation. Polyplexes made from the novel conjugates showed the best therapeutic effect (Fig. 6).

CONCLUSIONS

A novel polymeric carrier for tumor-targeted delivery of cytotoxic double-stranded RNA poly(I:C) was synthesized based on linear 22 kDa polyethylenimine (LPEI). EGF receptor targeting and effective killing of EGFR overexpressing tumor cells both *in vitro* and *in vivo* in tumor-bearing mice was made possible by selecting EGF as targeting ligand and attaching it to LPEI via a bifunctional PEG linker of optimum molecular weight (2 kDa PEG). More detailed analysis of the biological properties and therapeutic efficacy in tumor models will be performed in subsequent studies.

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