Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery

Martin Meyer, Alexander Philipp*, Reza Oskuee*, Claudia Schmidt[+] and Ernst Wagner**

* Pharmaceutical Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität München, Butenandtstr. 5 – 13, 81377 München, Germany

[+] Department of Physics, Ludwig-Maximilians-Universität München, Geschwister-Scholl-Platz 1, 80539 München, Germany

Email: ernst.wagner@cup.uni-muenchen.de

SUPPORTING INFORMATION

Experimental procedures

I. Materials

Branched polyethylenimine (PEI; average MW = 25kDa; catalog number 40,872-7; lot number 07112DF, polydispersity index 3.4), Poly-L-lysine-HBr (PLL; degree of polymerization = 153, MW = 32000; catalog number P2636, lot number 085K5100, polydispersity index 1.2), succinimidyl 3-(2-pyridyldithio) propionate (SPDP), 2,3-dimethylmaleicanhydride (DMMAn) and succinicanhydride (Succ) were obtained from Sigma-Aldrich (Munich, Germany). Cysteine-modified melittin (Mel) was obtained from IRIS Biotech GmbH (Marktredwitz, Germany) and 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. Succinimidyl propionate monomethoxy polyethylene glycol (SPA-mPEG, molecular weight 5000, catalog number 85969, lot number 071049/1) was purchased from Fluka (Buchs,

Switzerland). Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) was produced with the Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany) according to the manufacturer recommendations. Ready to use siRNA duplexes were purchased from MWG-Biotech (Ebersberg, Germany) namely, luciferase-siRNA: GL3 luciferase duplex: 5'-CUUACGCUGAGUACUUCGA-3'; control-siRNA: non-specific control duplex IX with similar GC content as anti-luciferase-siRNA and Cy5-siRNA: Cy5-labeled GL3 luciferase duplex: 5'-Cy5-CUUACGCUGAGUACUUCGA-3'. Cell culture media, antibiotics, and fetal calf serum were purchased from Invitrogen (Karlsruhe, Germany). Formulations for nucleic acid delivery were prepared in HBG (HEPES-buffered glucose solution; 20mM HEPES, 5% glucose, pH 7.4).

II. Conjugate synthesis

Synthesis of PEG-modified PLL

PLL (1.25µmol , 40 mg PLL hydrobromide, corresponding to 25.7 mg PLL free base) in 2 mL 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 dimethyl sulfoxide (DMSO). After 2 h at room temperature (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 3.0M NaCl in 20 mM HEPES pH 7.4. The fractions containing PLL-PEG were pooled, dialyzed against water and lyophilised. The degree of modification of PLL with PEG was determined by proton NMR. ^{1}H NMR spectra were recorded on a Jeol JNMR-GX500 (500 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) and refer to the solvent as internal standard (D_2O at 4.8 ppm). PLL-PEG: 1.26 – 1.82 (-CH2- ; PLL); 2.96 (-CH2-

N-; PLL); 4.27 (-NCHR-COO-); 3.66 (-CH2-CH2-O-; PEG). The content of PEG was calculated from the proton integrated values of PEG (-CH2-CH2-O-, 3.66 ppm) and the polylysine backbone protons (-NCHR-COO-, 4.27 ppm) and from the molecular weight values of the polymers and PEG given by suppliers. The PLL content was measured by trinitrobenzenesulfonic acid (TNBS) assay at 405 nm as described below.

Synthesis of PEG-modified PEI

Synthesis of PEI-PEG was carried out analogously as described above, starting with 31.3 mg (1,25 µmol) PEI in 2 ml buffer and mPEG-SPA (2,5 µmol, 12,5 mg). In case of PEI-PEG the content of PEG was calculated from the proton integrated values of PEG (-CH2-CH2-O-, 3.66 ppm) and the PEI protons (-CH2-CH2-NH-, 2.5 – 3.1 ppm). PEI content was measured by trinitrobenzenesulfonic acid (TNBS) assay at 405 nm as described below.

Synthesis of 3-(2-pyridyldithio)-propionate-modified PLL-PEG

PLL-PEG (0.313 µmol, containing 6.45 mg PLL) in 2 mL buffer (20 mM HEPES, pH 7.4) was mixed with SPDP (3.8 µmol, 1.19 mg) dissolved in 200 µl DMSO. After 2 h at RT PLL-PEG with pyridyldithio-propionate-linkers (PLL-PEG-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 PLL-PEG-PDP were pooled, aliquots were snap frozen in liquid nitrogen and stored at − 80 °C. PLL content of the fractions was measured by TNBS assay at 405 nm. The degree of modification with dithiopyridine linker was determined spectrophotometrically by release of pyridine-2-thion. 10µl of the sample was added to 140µl water and blank absorbance at 343 nm was measured. Subsequently 5µl of the reducing agent dithiothreitol (0,5 µmol, 77µg; diluted in

water) was added and release of pyridine-2-thion at 343 nm (molar absorptivity = 8080 M^{-1} cm^{-1}) was measured. The PLL-PEG-PDP conjugate had a molar ratio of PLL/PEG/PDP of approximately 1/1/8.

Synthesis of 3-(2-pyridyldithio)-propionate-modified PEI-PEG

PEI-PEG-PDP was synthesized analogously as described above, starting with 0,313 µmol (7.83 mg) PEI in 1,84 ml buffer. PEI content of the purified product was measured by TNBS assay. The degree of modification with dithiopyridine linker was determined spectrophotometrically by release of pyridine-2-thion as described above. The conjugate had a molar ratio of PEI/PEG/PDP of approximately 1/1.5/11.

Synthesis of DMMAn-melittin-modified PLL-PEG and PEI-PEG

Mel peptide (1.38 µmol, 4 mg) was dissolved in 400µl of 100 mM HEPES and 125 mM NaOH and mixed with 1000µl ethanol containing 15.8 µmol (2 mg) DMMAn by rapid vortexing under argon for 0.5 h following concentration and purification via ultrafiltration (Vivascience, Vivaspin 2, MWCO 2000 HY). 1.38 µmol of the acylated melittin was mixed under argon with 1.06 ml PLL-PEG-PDP (116 nmol PLL, 2.39 mg PLL, molar ratio of PLL/PEG/PDP of approximately 1/1/8) diluted in 2M guanidine hydrochloride, 0.5 M NaCl, 20 mM HEPES, pH 8. After 2 h at RT released pyridine-2-thion was measured at 343 nm to determine the extent of the reaction. The degree of modification was determined at 343 nm by release of pyrine-2-thione from residual PDP linkers after reduction with dithiothreitol.

PLL-PEG-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 0.5M NaCl, 20 mM HEPES, pH 8. The flow rate was 0.5 mL/min. The void fractions containing

PLL-PEG-DMMAn-Mel (molar ratio of PLL/PEG/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.

PEI-PEG-DMMAn-Mel conjugate was synthesized analogously, starting with 1.34 ml PEI-PEG-PDP (83.6 nmol PEI, 2.1 mg PEI, molar ratios PEI/PEG/PDP of approximately 1/1.5/11) yielding in PEI-PEG-DMMAn-Mel conjugate with a molar ratio of PEI/PEG/DMMAn-Mel of approximately 1/1.5/8.

Synthesis of succinic anhydride-melittin-modified PEI-PEG

Mel peptide (1.38 µmol, 4 mg) was dissolved in 400µl of 100 mM HEPES and 125 mM NaOH and mixed with 1000µl ethanol containing 15.8 µmol (1,58 mg) succinicanhydride by rapid vortexing under argon for 0.5 h following concentration and purification via ultrafiltration (Vivascience, Vivaspin 2, MWCO 2000 HY). Subsequent coupling to PEI-PEG-PDP and purification of PEI-PEG-Succ-Mel was carried out analogously to the PLL-PEG-DMMAn-Mel conjugate procedure as described above but using pH 7.4 buffers.

Synthesis of melittin-modified PLL-PEG and PEI-PEG

Mel peptide (1.38µmol, 4 mg) was dissolved in 400 µl of 0.5 M NaCl, 20 mM HEPES, pH 7.4 and mixed with 1060 µl PLL-PEG-PDP (116 nmol PLL, 2,39 mg PLL, molar ratio of PLL/PEG/PDP of approximately 1/1/8) diluted in the same buffer under argon. After 2 h at RT released pyridine-2-thion was measured at 343 nm to determine the extent of the reaction and degree of modification. Subsequent purification was carried out as described for PLL-PEG-DMMAn-Mel but using pH 7.4 buffers.

PEI-PEG-Mel conjugate was synthesized analogously starting with 1.34 ml PEI-PEG-PDP (83.6 nmol PEI, 2.1 mg PEI, molar ratios PEI/PEG/PDP of approximately 1/1.5/11).

TNBS assay

The concentration of PLL and PEI was measured by trinitrobenzenesulfonic acid assay. Standard PLL (or PEI) solutions and test solutions containing PLL 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 2.5µl of TNBS (75 nmol, 22µg; diluted in water) was added. TNBS reacts with primary amino groups to form colored trinitrophenylated derivatives. After 10 minutes at RT, absorption was measured at 405 nm using a microplate reader (Spectraflour Plus, Tecan Austria GmbH, Grödig, Austria).

III. Biophysical characterization

Ethidium bromide exclusion assay

The binding strength of the conjugates to siRNA was compared via ethidium bromide (EtBr) exclusion. The fluorescence of EtBr is significantly enhanced by nucleic acid intercalation. When a polycation binds to the nucleic acid, intercalated EtBr is displaced, and a reduction of fluorescence can be noticed. The fluorescence of a 20µg/mL solution of nucleic acid containing 400 ng/mL of EtBr in HBG was first measured (ex 510 nm, em 590 nm, slit width = 10 nm, Varian Cary Eclipse fluorescence spectrophotometer) and fluorescence was set to 100%. Aliquots of the conjugates were added stepwise to the nucleic acid EtBr solution and the decrease of fluorescence was measured indication interaction of the conjugate with the nucleic acid. Each data point represents the average of two measurements.

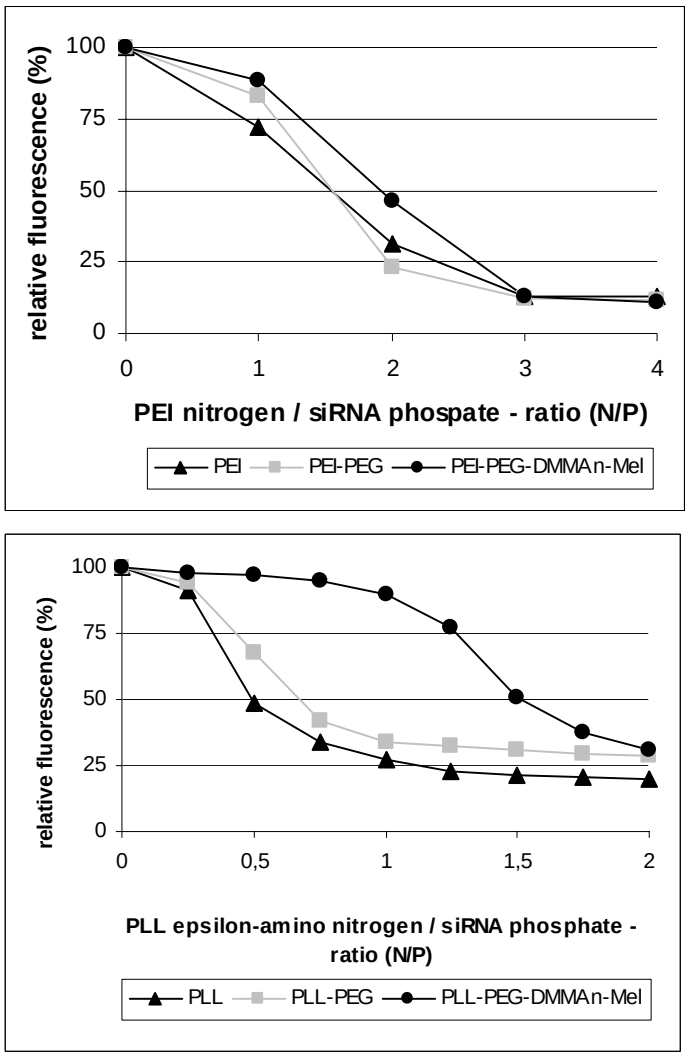


Figure S1. siRNA binding of conjugates measured by ethidium bromide exclusion assay.

Fluorescence correlation spectroscopy

FCS was employed to determine the size (hydrodynamic radius) of siRNA polyplexes. For FCS experiments siRNA labeled with Cy5 was utilized. FCS detects spontaneous intensity fluctuations resulting from fluorescently labeled molecules diffusing through a highly focused laser illuminated volume within the sample. The size of this volume is fixed by the confocal detection optics and the excitation profile of the focused laser beam and characterized by calibration measurements against a standard of a known diffusion constant. The raw signal, the time dependent fluorescence intensity fluctuations, is time-autocorrelated to obtain dynamic information on the fluorescently labeled molecules. For free Brownian diffusion of one identical species of particles, the autocorrelation function is given by

$$G(\tau) = 1 + \frac{1}{N} \frac{1}{1 + \frac{\tau}{\tau_D}} \frac{1}{\sqrt{1 + \frac{\tau}{S^2 \tau_D}}} \quad (1)$$

Where N represents the number of particles in the illuminated volume and $S = \frac{z_0}{r_{xy}}$ the structure parameter, the ratio of axial to radial dimensions of the focused laser beam which is a measure for the dimensions of the focal volume. The diffusion time τ_D of a particle through the illuminated focal volume with radius r_{xy} is related to the translational diffusion coefficient D by

$$\tau_D = \frac{r_{xy}^{\ 2}}{4D} \quad (2)$$

Additional intensity fluctuations can occur resulting from intramolecular processes like transitions to triplet states. If these processes are well separated in the time domain the autocorrelation function can be written as

$$G(\tau) = 1 + g_{triplett}(\tau) \times \frac{1}{N} \frac{1}{1 + \frac{\tau}{\tau_D}} \frac{1}{\sqrt{1 + \frac{\tau}{S^2 \tau_D}}} \quad (3)$$

with $g_{triplett}(\tau) = 1 + \frac{T_{triplett}}{1 - T_{triplett}} \exp\left(-\frac{\tau}{\tau_{triplett}}\right)$

where $T_{triplett}$ denotes the triplett fraction and τ_{triplett} the characteristic triplett decay time.

For fluorescently labeled spherical particles with dimensions smaller than the illuminated focal volume ($R \ll r_{xy}$) diffusing through a homogeneous solvent consisting of small molecules ($R \gg r_{solvent}$) the hydrodynamic radius R_H can be calculated from the Stokes-Einstein relation

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (4)$$

D denotes the translational diffusion coefficient of the particle, k_B the Boltzmann constant, T the temperature and η the solvent viscosity.

FCS Experiments

FCS experiments were performed using a commercially available FCS setup (Carl Zeiss, Jena, Germany), consisting of the Confocor 2 module and an Axiovert 200 inverted microscope equipped with a Zeiss C-Apochromat 40x, N.A. 1.2 water immersion objective. The illumination source was a HeNe Laser (633nm, 5mW) with emission passed through a 650nm long-pass before detection. All FCS measurements were performed at room temperature. Samples were pipeted directly into eight-well Lab-Tek chambers (Nalge Nunc International, Rochester, NY). Determination of the focal volume was established via calibration against an aqueous solution of 10nM Cy5 before each data acquisition. For each sample 10 measurements with sampling time of 30s were performed. The measured autocorrelation curves were fitted (Zeiss ConfoCor2 software package) with equation *(3),* the resulting diffusion time τ_D was used to calculate the hydrodynamic radius R_H of the siRNA polyplexes via equations (2) and (4).

IV. Cell culture, pDNA and siRNA transfections and cell viability assay

Cell culture

Cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere. Neuro2A murine neuroblastoma cells (ATCC CCL-131) and Neuro2A-eGFPLuc cells were cultured in DMEM (1 g/L glucose) containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM glutamine.

Formulation of transfection complexes

Formulations for siRNA delivery were prepared as follows: first 2µg siRNA and adequate amounts of the PEI- or PLL-conjugates using various polycation / siRNA ratios (w/w) 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 30min at room temperature to form the siRNA polyplexes that were used for transfection experiments. pDNA polyplexes were prepared similar in HBG at a pDNA concentration of 20µg/mL during polyplex formation.

pDNA was condensed with PEI or PEI-conjugates at a molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) of 6. pDNA was condensed with PLL or PLL-conjugates with a molar ratio of PLL epsilon-amine nitrogen to DNA phosphates of 2.

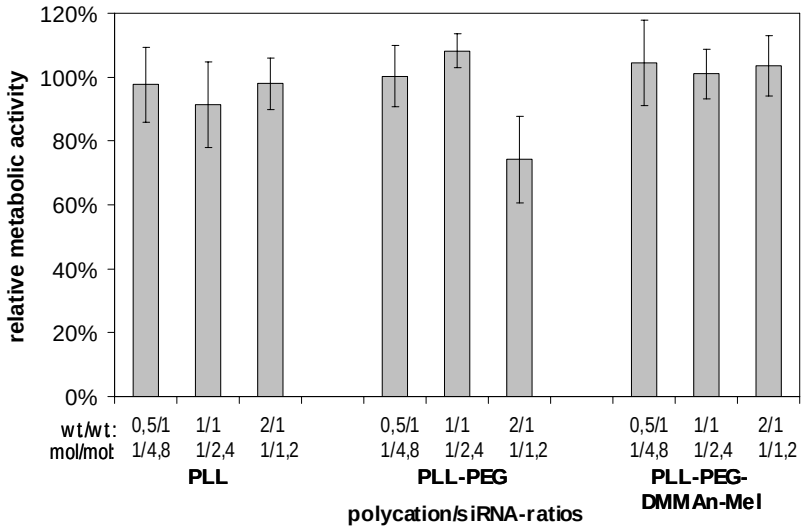
Luciferase siRNA transfection

All experiments were performed in stably transfected Neuro 2A-eGFPLuc cells. Cells were seeded in 96-well plates (TPP, Trasadingen, Switzerland) using 5000 cells per well 24 h prior to transfection. Transfection complexes containing 500 ng siRNA were then added to cells in 100 µl culture medium containing 10% serum, 100 U/ml penicillin and 100 µg/ml streptomycin (final

siRNA-concentration 367 nmol / liter). 48 h after initial transfection medium was removed and cells were lysed in 50µl Promega cell lysis solution to measure the gene expression as described below. Transfections were also performed with a non-specific control siRNA to distinguish between specific gene silencing and unspecific knockdown of protein expression due to carrier toxicity. Qualitative judgement on the toxicity of the conjugates was made by diminution in luciferase expression upon delivery of the non-specific control siRNA compared to the luciferase expression from the same number of cells that were not exposed to the carrier.

Metabolic activity assay

48 hours after initial transfection with siRNA polyplexes cell viability was measured. Metabolic activity of each well was determined using a methylthiazoletetrazolium (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. After incubation for 2 h at 37 ◦C, the medium was removed and cells were frozen for 2 hours (-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 (HBG)-treated control cells. Values of metabolic activity are presented as means ± SD of quintuplicates (Figure S2)



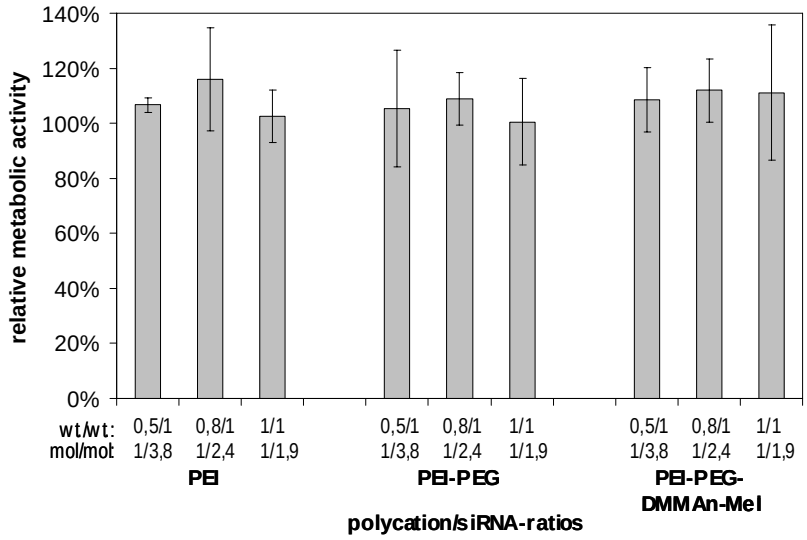


Figure S2. Metabolic activity of Neuro 2A-eGFPLuc cells 48 hours after initial siRNA transfection

Luciferase reporter gene expression

Cells were plated in 96 well plates at a density of 10000 cells (Neuro 2A) per well 24 h prior to transfection. The polyplexes with 200ng of pDNA (pCMVLuc) were added to the cells in 100 µl culture medium containing 10% serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The transfection medium was replaced after 3 h by 100 µl fresh cell culture medium. 24 h after initial transfection medium was removed and cells were lysed in 50µl 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 an 20µl 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. Two nanogram of recombinant luciferase (Promega, Mannheim, Germany) corresponded to 10^7 light units.

Additional transfection data

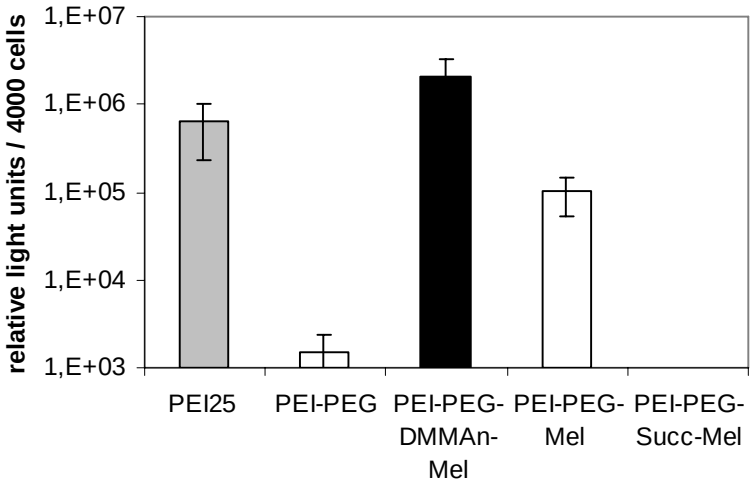


Figure S3. Gene transfer activity on Neuro 2A cells of PEI-based polyplexes (N/P 6) measured by luciferase reporter gene assay. Succinicanhydride modified melittin shows no lytic activity. In contrast to DMMAn-Mel the succinicanhydride group can not be cleaved upon acidification, which results in a loss of gene transfer activity of PEI-PEG-Succ-Mel.

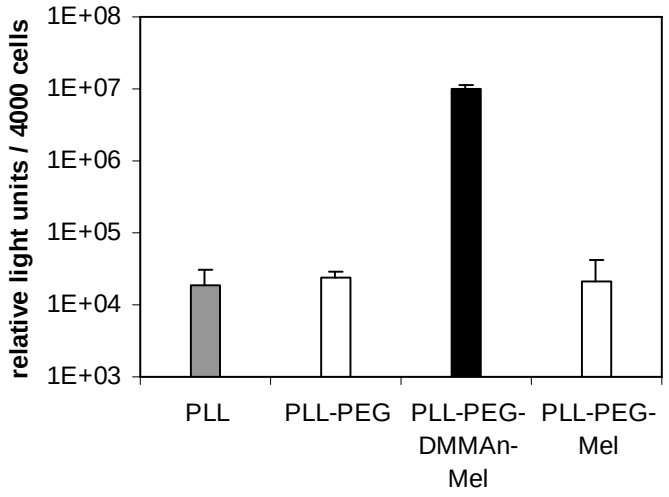


Figure S4. Gene transfer activity on Neuro 2A cells of PLL-based polyplexes (N/P 2) measured by luciferase reporter gene assay.