Pharmaceutical Research, Vol. 25, No. 12, December 2008 (© 2008)
DOI: 10.1007/s11095-008-9700-6

Research Paper

An Acid Sensitive Ketal-Based Polyethylene Glycol-Oligoethylenimine Copolymer Mediates Improved Transfection Efficiency at Reduced Toxicity

Veronika Knorr,[1] Manfred Ogris,[1] and Ernst Wagner[1,2]

Received June 13, 2008; accepted July 30, 2008; published online August 27, 2008

Purpose. Dynamic PEG-polycation copolymers that release PEG and degrade into small fragments after cell entry might present efficient and biocompatible gene carriers.
Methods. PEG-OEI-MK was synthesized by copolymerization of 5 kDa polyethyleneglycol (PEG) and 800 Da oligoethylenimines through acid-degradable acetone-bis-(*N*-maleimidoethyl)ketal linkers (MK). To evaluate any benefit of the reversible over stable linkage, also the corresponding pH-stable analog, PEG-OEI-BM, was synthesized *via* ether linkages. Luciferase and GFP expression plasmids were used for transfections, *in vivo* biocompatibility was evaluated by intravenous application of polymers in Balb/c mice.
Results. PEG-OEI-MK showed efficient DNA binding as analyzed by ethidium bromide exclusion, resulting in formation of polyplexes with sizes around 100 nm and surface charges of below 5 mV zeta potential. This surface shielding of PEG-OEI-MK polyplexes remained stable at neutral pH 7.4, while polyplexes deshielded and aggregated at pH 5 within 15–30 min. Cell culture experiments demonstrated reduced polymer toxicity compared to the non-PEGylated OEI-MK. Transfection experiments demonstrated reduced gene expression of PEG-OEI-BM compared with the non-PEGylated analog OEI-BM, whereas the pH-reversible polymer PEG-OEI-MK mediated a significant increased transfection efficiency over the non-PEGylated OEI-MK.
Conclusions. PEG-OEI-MK mediated the highest gene transfer at lowest cytotoxicity levels and also best *in vivo* biocompatibility.

KEY WORDS: gene transfer; ketal; nonviral; PEGylation; pH-sensitive.

INTRODUCTION

Due to their advantages concerning low immunogenicity, flexibility of incorporating nucleic acid constructs for different type and size, and the ease of handling, nonviral vectors are an attractive alternative to viral systems. Recent biodegradable polymer systems (1–15) are trend-setting, as they show much better biocompatibility than non-degradable analogs. Most recently we reported the generation of novel acetal linked oligoethylenimines that may undergo acid-catalyzed hydrolysis in the endosome (16). The pH-dependent degradation profiles displayed a short half-live of 3 min at pH 5.0 and an extended half-live of 5 h at physiological pH 7.4 and 37°C. DNA polyplexes formed with these degradable polymers showed high transfection efficiency and improved toxicity profiles compared to their acid-stable counterparts. Like many other plain polycation-based polyplexes, the transfection-competent particles displayed a positive surface charge (zeta potential +30 mV); this presents an undesired source for unspecific interactions with blood components and non-target cells as well as toxicity (17,18). The introduction of polyethylene glycol (PEG) chains or other hydrophilic neutral polymers has previously been demonstrated to reduce surface charge and toxicity of polyplexes (19–21). Although in few instances PEGylation also could improve gene transfer efficiency (22), in most cases stable PEGylation of polyplexes strongly reduces transfection efficiency (19–21). The use of pH-reversible (23–29) and other reversible (30) PEG shields has been demonstrated as a very encouraging solution to overcome the 'PEG-dilemma' (31).

[1] Pharmaceutical Biology-Biotechnology, Center for Drug Research and Center for NanoScience, Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, 81377, Munich, Germany.
[2] To whom correspondence should be addressed. (e-mail: ernst.wagner@cup.uni-muenchen.de)

ABBREVIATIONS: AP, alkaline phosphatase; AST, aspartate aminotransferase; BM, 1,8-bis-maleimidodiethyleneglycol; DCM, dichloro methane; EtBr, ethidium bromide; FCS, fetal calf serum; HBG, Hepes buffered glucose (5% (*w*/*v*) glucose, 20 mM Hepes, pH 7.4); 0.5× HBS, Hepes buffered salt (2.5% glucose (*w*/*v*), 75 mM NaCl, 20 mM Hepes, pH 7.4); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; MFI, mean fluorescence intensity; MK, 2,2-bis (*N*-maleimidoethyloxy) propane; m.w., molecular weight; Mw, molecular weight by weight; NaOAc, sodium acetate; OEI800, oligoethylenimine with an average molecular weight of 800 Da; PEG5K-SH, methoxy poly(ethylene glycol) thiol, average molecular weight 5 kDa; PEI25K, branched polyethylenimine with an average molecular weight of 25 kDa; PEI22K, linear polyethylenimine with an average molecular weight of 22 kDa; RLU, relative light units; s.d., standard deviation; U/l (37°C), units per liter plasma, measured at 37°C.

In the current article we combine the strategy of a pH-degradable polycationic gene carrier with a pH-sensitive PEG shield. We report on the syntheses and biophysical characterization of the acid-degradable block copolymer PEG-OEI-MK (assembled *via* ketal containing linkages) and its non-degradable analog PEG-OEI-BM (assembled *via* ether group containing linkages), and we compare the polymers concerning their *in vitro* transfection properties and *in vitro* and *in vivo* biocompatibility with their non-PEGylated analogues.

MATERIALS AND METHODS

Materials

Water was used in purified deionized form. Dimethylsulfoxide purissimum (DMSO) and dichloromethane purissimum (DCM) were obtained from Fluka (Fluka Chemie GmbH, Deisenhofen, Germany). Branched oligoethylenimine with an average molecular mass of 800 Da (OEI800), catalogue number 40819-100ML, batch # 08831KD, was purchased from Sigma-Aldrich (Munich, Germany), 1,8-*bis*-maleimidodiethyleneglycol (BM) was purchased from Pierce Biotechnology, Inc., Rockford, USA. 2,2-Bis(*N*-maleimidoethyloxy) propane (MK) was obtained from Organix Inc., Woburn, Massachusetts, USA. Methoxy poly(ethylene glycol) thiol, average molecular weight 5,000 (PEG5K-SH) was purchased from Nektar Therapeutics, Huntsville Alabama. Linear PEI of 22 kDa (PEI22K) was synthesized by acid-catalyzed deprotection of poly(2-ethyl-2-oxazoline; 50 kDa, Aldrich) in analogous form as described (32). Syntheses of the PEG-free polymer analogs OEI-BM and OEI-MK were carried out as described in (16) starting with OEI800. Spectra/Por dialysis membranes molecular mass cut off 3.5 kDa were purchased from Spectrum Laboratories Inc., Rancho Dominguez, CA, USA. Cell culture media, antibiotics, fetal calf serum (FCS) were purchased from Life Technologies (Karlsruhe, Germany). Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) described in Plank *et al.* (33) was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany).

Polymer Syntheses

PEG-OEI-BM

For synthesis of the pH-stable polymer PEG-OEI-BM, a solution of PEG5K-SH (14.2 mg; 2.5 μmol, 333 mg/ml in DCM) was added dropwise and under vortexing to a 19-fold excess of BM (15.4 mg; 50 μmol, 50 mg/ml in DMSO). After mixing the components thoroughly, the solution was added dropwise and under vortexing to OEI800 (38 mg; 47.5 μmol, 400 mg/ml in DMSO) and reacted for 22 h at room temperature under constant shaking. Prior to purification, the reaction mixture was diluted 1:4 with 1 M Hepes buffer pH 7.5 containing 4 M sodium chloride. Then dialysis was carried out at 4°C in 20 mM Hepes buffer pH 7.5 containing 0.25 M sodium chloride using a Spectra/Por membrane (molecular mass cut off 3.5 kDa). After 4 h the buffer was substituted for water; water was then replaced twice by fresh water. After total dialysis duration of 26 h, the purified product was lyophilized and stored at −80°C (yield 68% based on OEI in polymer/OEI input). According to NMR analysis, the molar ratio of PEG to BM to OEI800 in the product after purification was 0.05/1.3/1 which only slightly differs from the input ratio of 0.05/1/0.95.

^{1}H NMR (500 MHz, D_2O): δ=3.73 (*t*, 4H, NCH_2CH_2O), 3.67 (*s*, 472H OCH_2CH_2O of PEG ethylenes), 3.67 (*s*, 4H, OCH_2CH_2O linker ethylene), 3.60 (*t*, 2H, $COCHCH_2$ of linker succinimide), 3.58–2.35 (br *m*; 72H NCH_2 of OEI ethylenes; 4H NCH_2CH_2O of linker ethylenes and 4H $COCH_2CH$ of linker succinimide).

PEG-OEI-MK

For the synthesis of acid-degradable polymer PEG-OEI-MK, a solution of PEG5K-SH (14.2 mg; 2.5 μmol, 333 mg/ml in DCM) was added dropwise and under vortexing to MK (16.0 mg; 50 μmol, 50 mg/ml in DMSO). After mixing the components thoroughly, the solution was added dropwise and under vortexing to OEI800 (38 mg; 47.5 μmol, 400 mg/ml in DMSO) and reacted for 22 h at room temperature under constant shaking. Quenching, purification and lyophilization were performed as described above for PEG-OEI-BM (yield 43% based on OEI in polymer/OEI input). According to NMR analysis, the molar ratio of PEG to MK to OEI800 in the product after purification was 0.08/1.09/1 as compared to the input ratio of 0.05/1/0.95.

^{1}H NMR (400 MHz, D_2O): δ=3.86–2.30 (br *m*; 472H OCH_2CH_2O of PEG ethylene; 72H NCH_2 of OEI ethylenes; 14H NCH_2CH_2O of linker ethylene and $COCHCH_2$ of linker succinimide), 1.45–1.25 (*m*, 6H, CH_3 linker).

^{1}H NMR Analyses

^{1}H NMR spectra were recorded on a Jeol JNMR-GX400 (400 MHz) or on a Jeol JNMR-GX500 (500 MHz) spectrometer. Chemical shifts are reported in ppm; data are reported as *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet; br = broad.

Polymer Quantification

Quantification of polymers was performed by a copper complex assay described in (34): 50 μl of copper-(II)-sulfate dissolved in 0.1 M sodium acetate (0.23 mg/ml) of pH 5.4 were mixed with either 50 μl of aqueous standard OEI800 dilutions of known concentrations (standard curve) or with 50 μl of the solution of the OEI-based polymers. The resulting Cu(II)/amine complexes were quantified by measuring the absorbance at 285 nm using a GENESYS™ 10 Series Spectrometer (Thermo Electron Corporation, Pittsford, NY).

Molecular Weight Determination by SEC

Gel filtration was performed as described in (16). Briefly, HR 10/30 columns containing Superdex 75 or a Superdex 200 material were used (GE Healthcare Bio-Sciences AB, SE-75184 Uppsala) in combination with an HPLC 600 controller equipped with a photodiode array detector 996 from Waters (Waters GmbH Eschborn, Germany). Column material was preconditioned with PEI25K prior to use and then washed

with 20 mM Hepes buffer pH 7.4 containing 0.25 M NaCl which was chosen also as running buffer. Chromatography was performed at a flow rate of 1 ml/min and sample detection was carried out at λ=225 nm. Calibration with standard polyethylenimines of known molecular weights was performed as indicated in (16).

Ethidium Bromide (EtBr) Exclusion Assay

Aliquots of the respective polymer were added sequentially to a DNA solution (20 μg/ml) in HBG (Hepes buffered glucose; 20 mM Hepes pH 7.4 plus 5% *w*/*v* glucose) containing 400 ng/ml EtBr, and the decrease of fluorescence was measured in a Varian Cary Eclipse fluorescence spectrometer (Varian, Mulgrave, Australia). EtBr/DNA fluorescence (λex 510 nm and λem 590 nm) was set to 100% prior to addition of polycation.

Formation of Polyplexes

Plasmid DNA (pDNA) was mixed with the polycation at indicated cation/pDNA (*c*/*p*) ratios (*w*/*w*). In these calculations, only the weight of the OEI fraction within a cationic polymer was counted (i.e. not the total weight of PEG-OEI polymers). For PEI22K, *c*/*p*=0.78 presents *N*/*P*=6. Polycation/DNA polyplexes were prepared as described in (35) at final DNA concentrations of 100 μg/ml for biophysical analysis on the Zetasizer and of 20 μg/ml for *in vitro* transfection experiments. Briefly, indicated amounts of pDNA and polycation were each diluted in HBG pH 7.4 and mixed rapidly by pipetting. Polyplexes were incubated at room temperature for 20 min prior to use.

Particle Size and Zeta Potential

Particle size and surface charge of polycation/pDNA complexes were measured by laser-light scattering using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For these measurements complexes (preparation in HBG pH 7.4 as described above) were diluted with the indicated buffer to a final DNA concentration of 10 μg/ml (total volume 1 ml): for measurements at physiologic pH, HBG or 0.5× HBS of pH 7.4 was used, while for measurements in acidic milieu 20 mM NaOAc buffer pH 5.0, containing 75 mM NaCl, was added. For kinetics measurements these diluted polyplexes were incubated at 37°C for the indicated time periods.

Cell Culture

B16F10 murine melanoma cells (kindly provided by I.J. Fidler, Texas Medical Center, Houston, TX) were cultured in DMEM (1 g of glucose/l) supplemented with 10% FCS (*v*/*v*) and 1% penicillin/streptomycin (*v*/*v*). The cells were grown at 37°C in 5% CO_2 humidified atmosphere.

Luciferase Reporter Gene Expression and Metabolic Activity of Transfected Cells

In vitro transfection experiments were performed on B16F10 cells. Two parallel transfection series were carried out in separate 96-well plates (TPP), one for the determination of reporter gene expression and one for the determination of metabolic activity. 24 h prior to transfection cells were seeded with a density of 5×10^3 in 200 μl of culture medium (DMEM supplemented with 1 g of glucose/l, 10% FCS (*v*/*v*) and 1% penicillin/streptomycin) per well. Immediately before transfection, medium was removed from the wells and 100 μl of a dilution of transfection complexes (200 ng pDNA/well) in culture medium were added to the cells. After 4 h of incubation at 37°C complex containing medium was replaced by 100 μl of fresh medium. Transfection efficiency was evaluated 24 h after treatment by measuring luciferase reporter gene expression using a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wilbad, Germany) as described previously (36). Values are given as relative light units (RLU) and represent the luciferase activity per 10^4 cells. 2 ng of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^7 light units.

Relative metabolic activity of cells was determined 24 h after transfection by the methylthiazoletetrazolium (MTT)/thiazolyl blue assay. 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, regarded as 100%.

Enhanced Green Fluorescent Protein Expression (EGFP) Study

Twenty-four hours prior to transfection B16F10 cells were seeded in 24-well plates with a cell density of 2×10^4 cells in 1 ml of culture medium (DMEM supplemented with 1 g of glucose per liter, 10% FCS (*v*/*v*) and 1% penicillin/streptomycin) per well. Just before transfection, medium was removed and replaced by 1 ml of a dilution of the transfection complexes (1 μg pEGFP-N1/well) in culture medium. After 4 h of incubation at 37°C the complex containing medium was replaced by 1 ml of fresh medium. Finally, after additional 20 h of incubation, the cells were washed with phosphate-buffered saline and harvested by trypsin treatment. Analysis was performed as described in (37) using a CyanADP flow cytometer (DakoCytomation, Kopenhagen, Denmark). GFP fluorescence was excited at 488 nm and emission was detected using a 530±40 bandpass filter. To exclude doublets and dead cells, cells were appropriately gated by forward/side scatter and pulse width. Per sample 1×10^4 gated events were collected; values are given as the average of triplicates of transfected cell cultures.

In Vivo Biocompatibility Study

Animal experiments were performed according to National Regulations and were approved by the Local Animal Experiments Ethical Committee. Female Balb/c mice were purchased from Janvier (Le Genest St Isle, France). Animals were housed in individually vented cages, food and water were provided *ad libitum*. For toxicity studies, polymers were dissolved in HBG and injected into the tail vain of the 8-week-old mice. Thereby the polymers were applied at amounts of 100 or 50 μg/20 g mouse, in an injection volume of 250 μl (The polymer amounts are calculated for the

amount of polycation in the polymers, the PEG content is not included in the mass declarations.). 48 h after polymer treatment, mice were killed and perfusion-fixed with formalin solution (4% paraformaldehyde in phosphate-buffered saline). Main organs (livers, kidneys, lungs) were resected and embedded in paraffin. Sections of 5 μm thickness were cut and stained with hematoxylin–eosin for histological investigations. Microscopic pictures were taken with a Framos Infinity 2-3C, CCD-camera on an Axiovert 200 inverted microscope (Carl Zeiss) using a ×40 LC Achroplan objective.

For determination of the liver-specific blood enzymes alkaline phosphatase (AP) and aspartate aminotransferase (AST), blood was collected by heart puncture immediately after sacrification using heparinized syringes. Samples were centrifuged at 3,000×*g* for 10 min at 4°C and the supernatants were collected for plasma analysis. AP and AST were measured using a kinetic UV test from Olympus (Olympus Life and Material Science, Hamburg, Germany).

Statistical Analysis

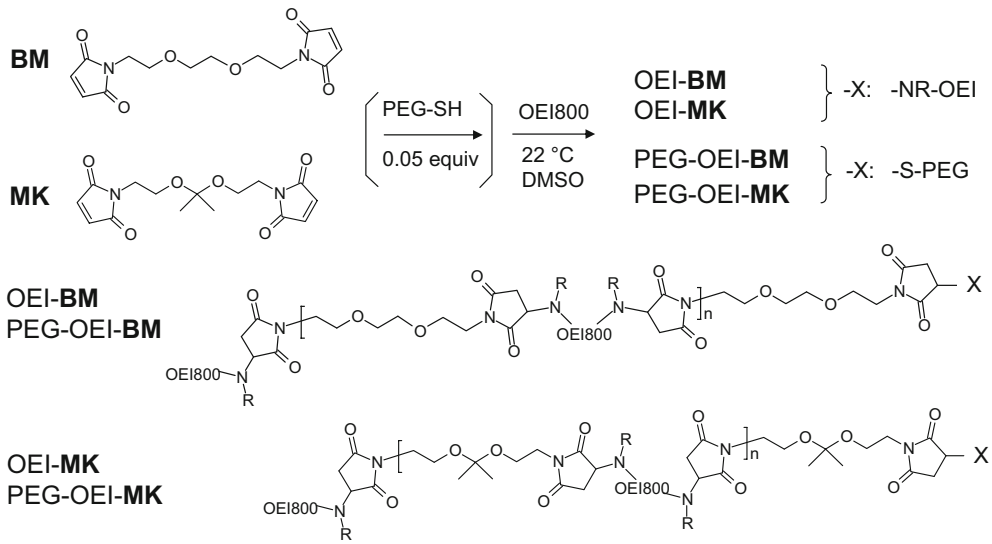
Results are presented as mean ± sd. Data were evaluated by variance analysis (ANOVA) using the Duncan test; *p*-values smaller than 0.05 were considered to be significant; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

RESULTS

Polymer Syntheses and Chemical Analysis

The syntheses are outlined in Scheme 1. To introduce PEG into pH-degradable OEI-MK or pH-stable OEI-BM polymers, thiol modified PEG of 5 kDa (PEG5K-SH) was first reacted with a 20-fold excess of linker MK or BM to completely convert it into a maleimido-linker containing PEG molecule, followed by the subsequent addition of OEI800. Molar ratios of PEG/linker/OEI800 in these one-pot reactions were 0.05/1/0.95. The non-PEGylated analogues OEI-MK and OEI-BM were synthesized at molar input ratios linker/OEI800 of 1/1 as previously described (16). The purification of all resulting polymers by dialysis using a 3.5 kDa molecular mass cut off (MWCO) membrane was carried out at 4°C and pH 7.5 to avoid hydrolysis of the ketal-containing polymers, followed by lyophilization and storage at −80°C. In the dialysis we used the same MWCO membrane for all polymers (PEGylated and non-PEGylated), as we subsequently wanted to compare the PEGylated polymers with the analogous non-PEGylated polymers. A possible contamination with non-PEGylated oligomers was accepted; in previous work (25,27) we deliberately used defined mixtures of PEGylated with non-PEGylated polymers. The PEG contents as well as linker to OEI ratios were determined by ^{1}H NMR analyses. For PEG-OEI-BM a proportion of 0.05/1.3/1 PEG/linker/OEI800 was found, representing one PEG molecule in average per 20 OEI molecules, while the ratio was 0.08/1.1/1 in case of PEG-OEI-MK, representing one PEG molecule in average per 13 OEI molecules. The slight differences between molar input ratios (PEG/linker/OEI800, 0.05 /1 /0.95) and obtained ratios in the purified polymers (PEG-OEI-BM: 0.05 /1.3 /1; PEG-OEI-MK: 0.08 / 1.1 /1) can be explained by the dialysis purification process, due to a preferential recovery of conjugates with slightly different composition than the input ratios.

Analysis of polymer size by SEC (Fig. 1) showed that PEG-OEI-BM and PEG-OEI-MK possess size distributions with two peak maxima, one in the range of 7 kDa and a second one in the range of 40 kDa (in case of PEG-OEI-BM) or 20 kDa (in case of PEG-OEI-MK). Both polymers, due to the additional presence of the 5 kDa PEG, show a shift toward the higher m.w. range as compared to the non-PEGylated analogues OEI-BM and OEI-MK. For PEG-OEI-BM the average Mw increased to 25 kDa compared to the PEG-free OEI-BM with average Mw of 15 kDa. The average Mw of OEI-MK was calculated to be 9 kDa, while for the PEGylated variant it was slightly higher at about 11 kDa.



Scheme 1. Syntheses of polymers OEI-MK and OEI-BM and their PEGylated variants. For syntheses of the PEGylated polymers, the linkers were reacted first in 19-fold molar excess with PEG-SH, before OEI800 was added. R: H or ethylenimine units.

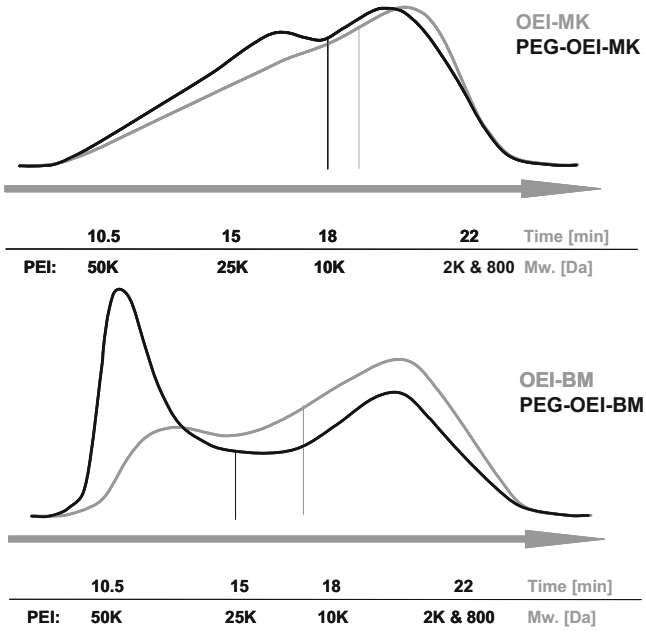


Fig. 1. Analysis of polymer molecular weight by SEC on Superdex 200. Calibration performed with standard polymers of known molecular weights and of comparable chemical structure (OEI800, PEI2K, PEI10K, PEI25K and PEI50K) is indicated on the *arrow* below the diagrams.

Biophysical Analysis of the Polymers

To investigate if PEGylation hampers DNA binding capacity of the polymers, EtBr exclusion assay was performed. In this assay the presence of PEG caused no (*c*/*p* ratios ≥5) or only marginal (*c*/*p*>0.5) impairment of DNA binding compared to the non-PEGylated polymers (Fig. 2).

Next, the effect of PEG shielding on polyplex size and surface charge was investigated. OEI-MK and OEI-BM conjugates with or without PEG were mixed with pDNA at several *c*/*p* ratios and diluted with HBG pH 7.4. Differences in particle size as well as in zeta potential were found for the PEGylated polyplexes compared to the PEG-free ones: particle stabilization against aggregation was detected upon PEGylation especially in the low *c*/*p* range; while non-PEGylated polyplexes were big in size at lower *c*/*p* ratios,

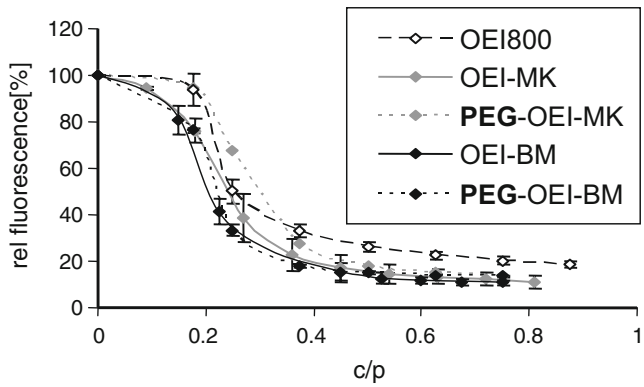


Fig. 2. Ethidium bromide exclusion assay in HBG, pH 7.4. Influence of PEGylation on DNA binding: PEG does not or only slightly (at very low *c*/*p* ratios) hamper binding ability of the polymers. *Each point* represents the mean ± SD of three experiments.

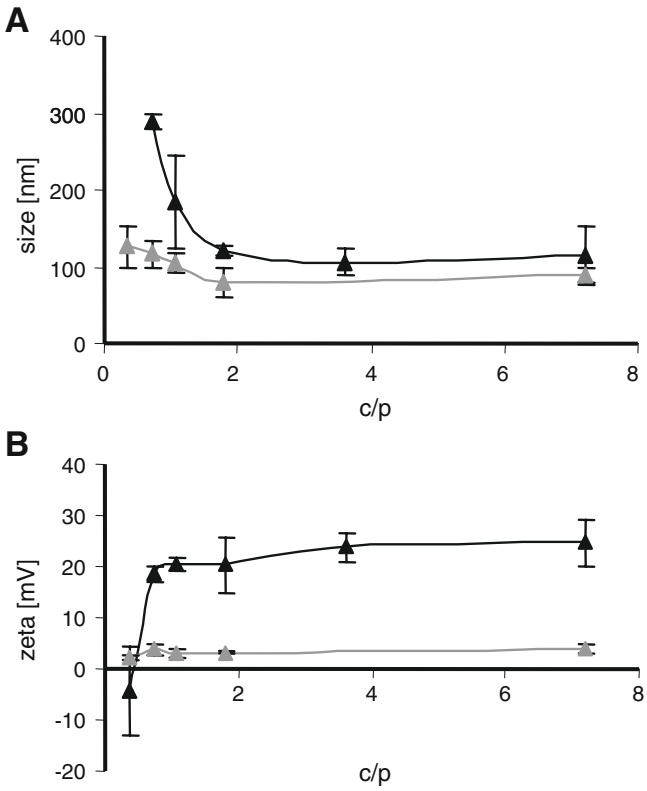


Fig. 3. Determination of particle size (**a**) and surface charge (**b**) in presence (PEG-OEI-MK) or absence (OEI-MK) of PEG in HBG pH 7.4. Particles were formed either with OEI-MK (*black triangles*) or with PEG-OEI-MK (*grey triangles*) and pDNA at different *c*/*p* ratios. Nearly neutral zeta potentials and stable small particle sizes of the PEGylated particles demonstrate the shielding effect of PEG-OEI-MK compared to non-PEGylated OEI-MK. *Each point* represents the mean ± SD of three experiments.

PEGylated particles possessed constant small sizes around 100 nm independent from the *c*/*p* ratio (Fig. 3a). The shielding influence of PEG became even more distinct when particles were analyzed for their surface charge. Here, PEGylated particles showed almost neutral zeta potentials over the whole *c*/*p* range tested. Surface charges of non-PEGylated polyplexes changed with the *c*/*p* ratio, from negative values at *c*/*p* of 0.25 up to above +20 mV for *c*/*p* 1 (Fig. 3b). These values do not change at higher *c*/*p* ratios when additional polymer is expected to be free and not bound to the polyplex particles (36). This study proves successful PEGylation of OEI-MK and OEI-BM and demonstrates that the chosen PEG content is sufficient to achieve surface shielding of PEG-OEI-MK and PEG-OEI-BM polyplexes.

Next the reversibility of PEG shielding was evaluated (Fig. 4). Polyplexes prepared in HBG at *c*/*p*=1 were diluted either with buffer of pH 7.4 or 5.0. The samples were then incubated at 37°C for different time periods and changes in particle size and charge were analyzed. While PEG-OEI-MK particles were stable at pH 7.4, in acidic medium these particles grew in size and finally aggregated; in parallel their zeta potential increased over time (Fig. 4a). These data demonstrate that the ketal-linked PEG allows shielding in physiological fluids but at the same time is acid-reversible, which is of interest for endosomal deshielding of polyplexes.

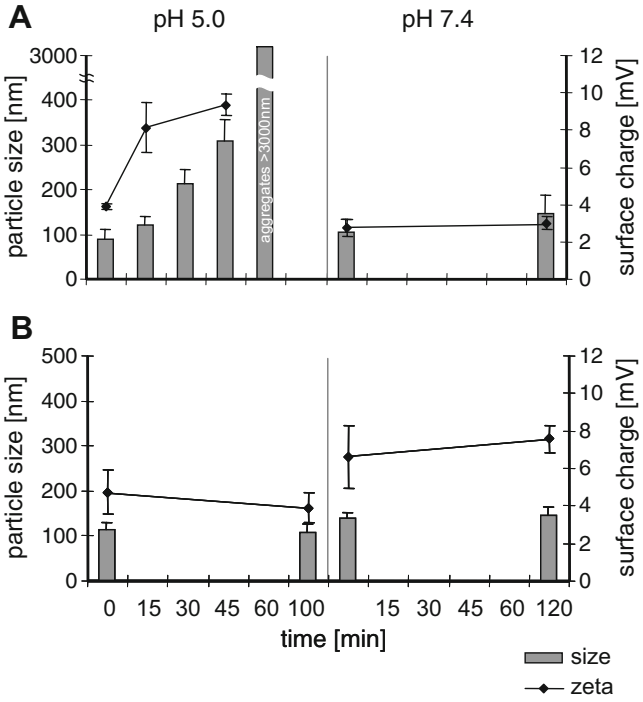


Fig. 4. Particle size and surface charge of PEG-OEI-MK (**a**) and PEG-OEI-BM (**b**) polyplexes (*c*/*p*=1) measured at different time points after incubation at 37°C. Measurements were performed either in 75 mM NaCl containing either 20 mM NaOAc pH 5.0 (*left panels*) or 20 mM Hepes pH 7.4 (*right panels*). Increasing values for particle size and surface charge over the time indicate deshielding, while constant values indicate stability of PEGylation. *Each point* or *bar* represents the mean ± SD of three independent experiments.

In contrast PEG-OEI-BM particles changed their characteristics neither at physiologic nor at acidic pH, attesting that in this case PEGylation is stable, independent from the existing pH (Fig. 4b).

In Vitro Transfection Results and Metabolic Activity of Transfected Cells

Transfection efficiency of the polymers as well as polyplex toxicity were evaluated *in vitro* on B16F10 cells. Therefore, PEG-OEI-MK was not only compared to non-PEGylated OEI-MK but also to the stable analogs OEI-BM and PEG-OEI-BM. The experiments demonstrated that not only the presence or absence of PEG had an impact on the transfection results, but also the nature of the PEGylation (stable or acid-labile) influenced transfection efficiency.

Independent of the stability of the linkage, PEGylation significantly improved compatibility of the polyplexes in B16F10 cells, especially at higher *c*/*p* ratios (Fig. 5a). Further it turned out that sensitivity to endosomal acidic conditions improved the biocompatibility: the degradable PEG-OEI-MK was significantly less toxic than the corresponding nondegradable PEG-OEI-BM polymer.

In terms of transfection efficiency (luciferase assay) stable PEGylation had a negative effect, thus reducing gene expression of OEI-BM remarkably (Fig. 5b). In contrast, for OEI-MK the reversible PEGylation even improved the transfection efficiency of the polymer. At higher *c*/*p* ratios (*c*/*p* 12.5) PEG-OEI-MK showed gene expression values comparable to those reached with the non-shielded, golden standard PEI22K. At *c*/*p* ratios of 10 and 12.5 the superiority

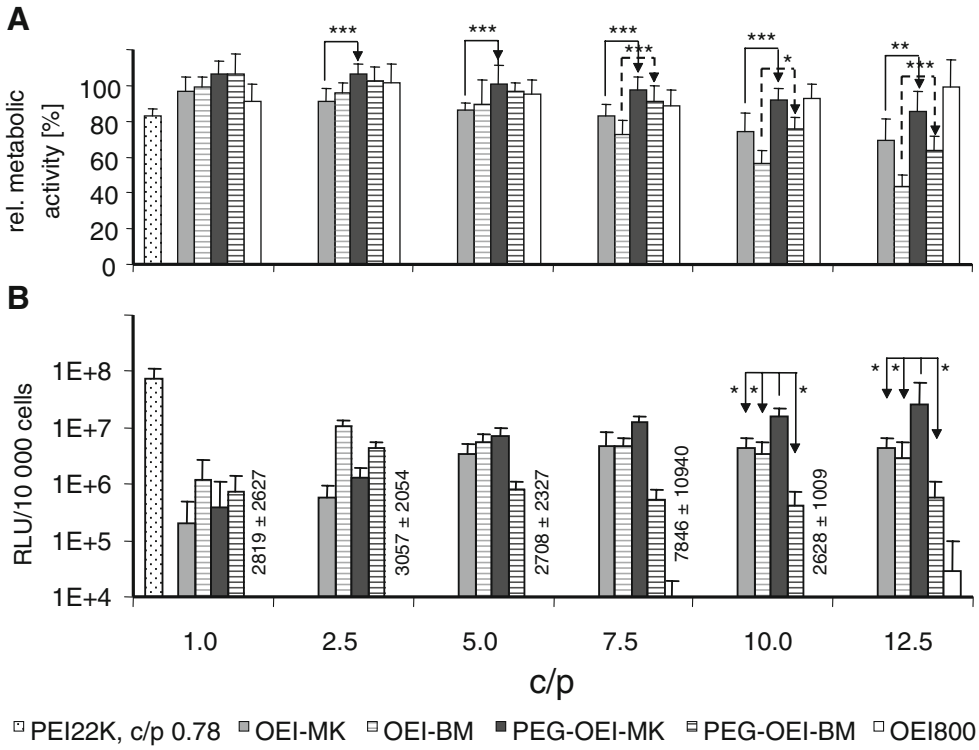


Fig. 5. Influence of PEGylation of OEI-MK and OEI-BM on transfection efficiency and polyplex toxicity in B16F10 cells. While stable PEGylation (PEG-OEI-BM) only improved polyplex compatibility but decreased transfection efficiency, acid-reversible PEGylation (PEG-OEI-MK) had positive effects on both features.

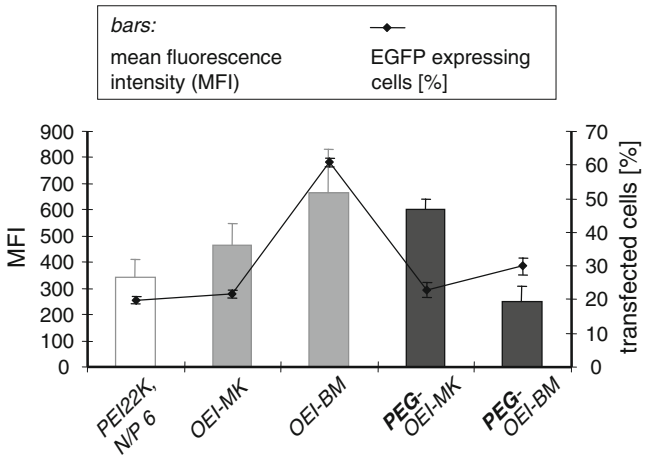


Fig. 6. Enhanced green fluorescent protein (EGFP) expression in B16F10 cells. Polyplexes were prepared at a *c/p* ratio of 10 (except PEI22K, which was prepared at its optimum *c/p* ratio of 0.78) in HBG. The diagram shows a representative experiment, each *diamond* or *bar* demonstrates the mean of a triplicate. *Bars* present mean fluorescence intensities (MFI) in arbitrary fluorescence units of the transfected cell population, *diamonds* present percent of GFP-fluorescent cells.

of PEG-OEI-MK was significant over all three other OEI800 based polymers (OEI-MK, OEI-BM and PEG-OEI-MK).

To further elucidate the differences in transfection properties of OEI-MK-based and OEI-BM-based polymers, as well as of the acid-reversible or acid-stable PEGylation technique, FACS analysis of pEGFP transfected B16F10 cells was performed (Fig. 6). PEI22K control polyplexes were again used at their optimal *c/p* ratio of 0.78 (i.e. N/P ratio of 6), while for the OEI-based polymers a *c/p* ratio of 10 was chosen. The number of transfected cells was highest for the non-modified BM-A1/1 polymer (~60%), followed by PEG-OEI-BM, PEG-OEI-MK, OEI-MK and finally PEI22K, which varied only slightly in their numbers of transfected cells (30–20%). One has to keep in mind that only viable cells are measured in this assay, while dead cells are not taken into account. Therefore, in contrast to the luciferase assay, the higher toxicity of BM polymers is not represented, which makes toxic polymers appear in a better light. Nevertheless, the study shows the positive effects of pH-reversible PEGylation on the transfection properties. Regarding the mean fluorescence intensity (MFI) per cell, stable PEGylation of OEI-BM resulted in a loss of reporter gene expression: while for OEI-BM a MFI of 666 was measured, for PEG-OEI-BM this value was only about 1/3 as high (MFI 252). Also the number of viable cells expressing EGFP decreased for about 50% upon the introduction of a stable PEG shield. In contrast, acid-reversible PEGylation of OEI-MK enhanced the MFI value from 466 (OEI-MK) to 600 (PEG-OEI-MK), and no decrease in the number of transfected cells was observed. Taken together, this study confirmed that transfection properties are affected positively by acid-labile PEGylation, while they become worse in the presence of stable PEG.

In Vivo Biocompatibility Studies

As the strategy of acid sensitive PEGylation seemed to be very promising concerning improved transfection efficiency and reduced polymer toxicity, the biocompatibility of the PEG-OEI-MK polymer was evaluated also *in vivo* in Balb/c mice. HBG-solutions of PEG-OEI-MK and its comparative polymers OEI-BM, PEG-OEI-BM and OEI-MK were administered to 8-weeks old female Balb/c mice *via* tail vain injection. As previously reported, treatment with 100 μg polymer/20 g mouse was lethal in case of OEI-BM (16). Therefore we reduced the dose to 50 μg, which however still induced the development of a fatty liver (Fig. 7a, upper left picture). PEGylation of the polymer resulted in an improved biocompatibility: the dosage of 50 μg /20 g mouse was well tolerated by all three animals and liver tissue was not affected pathologically (Fig. 7a, upper right picture). However, treatment with 100 μg of PEG-OEI-BM was survived only by one mouse out of three. This indicates that PEGylation improved polymer compatibility, but still considerable toxicity remains due to non-degradability of the polymer. Degradable OEI-MK in contrast, is far more biocompatible. The high 100 μg dosage (Fig. 7a, lower left picture) was tolerated by all three animals; only in one of them the onset of fatty liver was

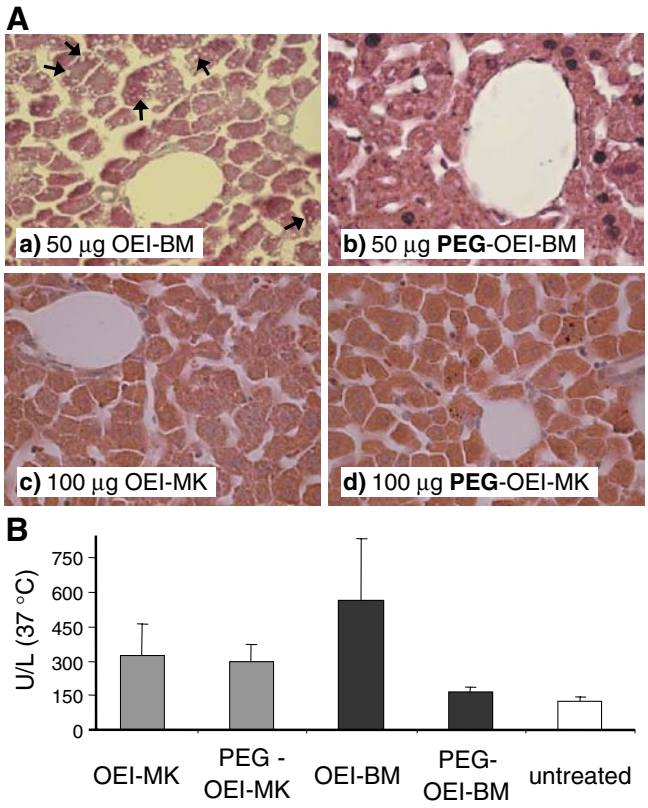


Fig. 7. *In vivo* study on toxicity of the free polymers in Balb/c mice. **a** Histological examination on liver tissue (hematoxylin–eosin staining) 48 h after i.v. administration of indicated amounts of free polymer per 20 g mouse. Development of a fatty liver (*black arrows* indicate lipid droplets in hepatocytes) after application of 50 μg of non-degradable OEI-BM polymer (*upper left picture*). Its PEGylated variant (PEG-OEI-BM, *upper right picture*) in contrast was well tolerated, no inclusion of lipid droplets in liver tissue occurred. Degradable OEI-MK and PEG-OEI-MK polymers (*lower left* and *right pictures*, respectively), applied even at dosages of 100 μg induced no or only marginal histopathological changes in the liver. **b** AST level in serum of mice treated with the indicated polymers at a dosage of 50 μg/20 g mouse. Number of mice for each treatment group: $n=3$.

detectable. For the PEGylated variant PEG-OEI-MK (Fig. 7a, lower right picture) the results were very similar, with again one mouse out of three which developed a fatty liver and two with completely healthy liver tissue. The other harvested organs (lung, kidneys) were not affected by the treatment with any of the polymers and did not show any histopathological changes. The results demonstrate that both, degradability and PEGylation contribute to a high compatibility of the polymers.

Additionally, AP and AST levels in the plasma of the mice were determined. In case of AP no significant differences could be detected in animals treated with OEI-MK, PEG-OEI-MK, OEI-BM or PEG-OEI-BM (dosage: 50 µg/20 g mouse). In contrast, the AST level (Fig. 7b) of OEI-BM-treated mice was significantly ($p<0.01$) enhanced over that of non-treated control mice, demonstrating destruction of liver tissue by this polymer. PEGylation of BM-A1/1, however, could reduce the AST level significantly ($p<0.05$). AST levels of mice treated with PEG-OEI-BM, OEI-MK or PEG-OEI-MK did not differ significantly from those of untreated control mice. This confirms the advantage of polymer degradability and PEGylation for an improved biocompatibility.

DISCUSSION

Nanoscaled nucleic acid/cationic polymer complexes (polyplexes) present a very interesting class of formulations for delivery of plasmid DNA, siRNA and related therapeutic nucleic acids (15,38–41). Especially the class of biodegradable and biocompatible polymers (1–14) has gained more and more interest during the last years. PEGylation of polycations is a common strategy to further improve biocompatibility of polyplexes (19,20) but has the drawback of reducing transfection efficiency at the same time. For high transfection levels it is essential that the endosomally trapped polyplexes are efficiently released into the cytosol. The required endosomal membrane destabilization is facilitated by several mechanisms including interaction of the positively charged polymers with the endosomal membrane which is hampered heavily by the presence of a PEG shield (31). This handicap however can be circumvented by reversibly linking the PEG onto the polycation. The concept is based on the generation of dynamic delivery formulations (42,43), where PEG molecules shield polyplexes during the circulation, but are cleaved after reaching the target cell by a defined biological trigger, and therefore do not hamper subsequent cellular transfection steps. The acidic endosomal pH presents such a specific biological trigger (31). It has already been demonstrated that transfection efficiency of PEI polyplexes could be restored by acid-cleavable PEG shielding in the past (25,27,29). In the current work, the introduction of a reversible PEG-shield into the polymer OEI-MK (16) was performed concomitantly with the synthesis of the polymeric carrier in a very simple procedure, thus avoiding additional purification and production steps. In contrast to the stable control polymer PEG-OEI-BM, where PEGylation lowered polymer toxicity but also diminished transfection efficiency as expected, the acid labile PEGylation in OEI-MK did not only reduce toxicity, but interestingly also improved transfection efficiency compared to the non-PEGylated OEI-MK polymer. This clearly outlines the benefit of reversible linkages for PEGylation over stable PEG conjugation. Intravenous application in Balb/c mice demonstrated the high biocompatibility of the PEGylated ketal-based polymer, encouraging further *in vivo* evaluation of tumor-targeted polyplexes based on this novel polymeric carrier.

ACKNOWLEDGMENTS

We thank Olga Brück for skillful assistance in preparing the manuscript, as well as Wolfgang Roedl and Anna Kulinyak for competent technical assistance. This work was funded by the LMU university, by the DFG projects SFB 486 'Nanoman' and SPP1230, the Nanosystems Initiative Munich (NIM), and the European Community FP6 network project 'GIANT'.

REFERENCES

1. J. Wang, H. Q. Mao, and K. W. Leong. A novel biodegradable gene carrier based on polyphosphoester. *J. Am. Chem. Soc.* **123**:9480–9481 (2001). doi:10.1021/ja016062m.
2. Y. B. Lim, S. M. Kim, H. Suh, and J. S. Park. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjug. Chem.* **13**:952–957 (2002). doi:10.1021/bc025541n.
3. M. L. Forrest, J. T. Koerber, and D. W. Pack. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. *Bioconjug. Chem.* **14**:934–940 (2003). doi:10.1021/bc034014g.
4. D. G. Anderson, D. M. Lynn, and R. Langer. Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. *Angew. Chem. Int. Ed. Engl.* **42**:3153–3158 (2003). doi:10.1002/anie.200351244.
5. J. J. Green, J. Shi, E. Chiu, E. S. Leshchiner, R. Langer, and D. G. Anderson. Biodegradable polymeric vectors for gene delivery to human endothelial cells. *Bioconjug. Chem.* **17**:1162–1169 (2006). doi:10.1021/bc0600968.
6. J. Kloeckner, E. Wagner, and M. Ogris. Degradable gene carriers based on oligomerized polyamines. *Eur. J. Pharm. Sci.* **29**:414–425 (2006). doi:10.1016/j.ejps.2006.08.002.
7. M. Thomas, J. J. Lu, C. Zhang, J. Chen, and A. M. Klibanov. Identification of novel superior polycationic vectors for gene delivery by high-throughput synthesis and screening of a combinatorial library. *Pharm. Res.* **24**:1564–1571 (2007). doi:10.1007/s11095-007-9279-3.
8. L. V. Christensen, C. W. Chang, W. J. Kim, S. W. Kim, Z. Zhong, C. Lin, J. F. Engbersen, and J. Feijen. Reducible poly(amido ethylenimine)s designed for triggered intracellular gene delivery. *Bioconjug. Chem.* **17**:1233–1240 (2006). doi:10.1021/bc0602026.
9. D. S. Manickam, and D. Oupicky. Multiblock reducible copolypeptides containing histidine-rich and nuclear localization sequences for gene delivery. *Bioconjug. Chem.* **17**:1395–1403 (2006). doi:10.1021/bc060104k.
10. Y. Lee, H. Mo, H. Koo, J. Y. Park, M. Y. Cho, G. W. Jin, and J. S. Park. Visualization of the degradation of a disulfide polymer, linear poly(ethylenimine sulfide), for gene delivery. *Bioconjug. Chem.* **18**:13–18 (2007). doi:10.1021/bc060113t.
11. J. J. Hoon, L. V. Christensen, J. W. Yockman, Z. Zhong, J. F. Engbersen, K. W. Jong, J. Feijen, and K. S. Wan. Reducible poly (amido ethylenimine) directed to enhance RNA interference. *Biomaterials*. **28**:1912–1917 (2007). doi:10.1016/j.biomaterials.2006.12.019.
12. C. Lin, C. J. Blaauboer, M. M. Timoneda, M. C. Lok, M. van Steenbergen, W. E. Hennink, Z. Zhong, J. Feijen, and J. F. Engbersen. Bioreducible poly(amido amine)s with oligoamine side chains: synthesis, characterization, and structural effects on gene delivery. *J. Control. Release*. **126**:166–174 (2008). doi:10.1016/j.jconrel.2007.11.012.

13. J. Kloeckner, S. Bruzzano, M. Ogris, and E. Wagner. Gene carriers based on hexanediol diacrylate linked oligoethylenimine: effect of chemical structure of polymer on biological properties. *Bioconjug. Chem.* **17**:1339–1345 (2006). doi:10.1021/bc060133v.
14. V. Russ, H. Elfberg, C. Thoma, J. Kloeckner, M. Ogris, and E. Wagner. Novel degradable oligoethylenimine acrylate ester-based pseudodendrimers for *in vitro* and *in vivo* gene transfer. *Gene Ther.* **15**:18–29 (2008). doi:10.1038/sj.gt.3303046.
15. S. Y. Wong, J. M. Pelet, and D. Putnam. Polymer systems for gene delivery—past, present, future. *Prog. Polym. Sci.* **32**:799–837 (2007). doi:10.1016/j.progpolymsci.2007.05.007.
16. V. Knorr, V. Russ, L. Allmendinger, M. Ogris, and E. Wagner. Acetal linked oligoethylenimines for use as pH-sensitive gene carriers. *Bioconjug. Chem.* **19**:1625–1634 (2008). doi:10.1021/bc8001858.
17. C. Plank, K. Mechtler, F. C. Szoka Jr., and E. Wagner. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther.* **7**:1437–1446 (1996). doi:10.1089/hum.1996.7.12-1437.
18. P. Chollet, M. C. Favrot, A. Hurbin, and J. L. Coll. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J. Gene Med.* **4**:84–91 (2002). doi:10.1002/jgm.237.
19. M. Ogris, S. Brunner, S. Schuller, R. Kircheis, and E. Wagner. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **6**:595–605 (1999). doi:10.1038/sj.gt.3300900.
20. P. Erbacher, T. Bettinger, P. Belguise-Valladier, S. Zou, J. L. Coll, J. P. Behr, and J. S. Remy. Transfection and physical properties of various saccharide, poly(ethylene glycol), and antibody-derivatized polyethylenimines (PEI). *J. Gene Med.* **1**:210–222 (1999). doi:10.1002/(SICI)1521-2254(199905/06)1:3<210::AID-JGM30>3.0.CO;2-U.
21. D. Oupicky, M. Ogris, K. A. Howard, P. R. Dash, K. Ulbrich, and L. W. Seymour. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* **5**:463–472 (2002). doi:10.1006/mthe.2002.0568.
22. M. Mannisto, S. Vanderkerken, V. Toncheva, M. Elomaa, M. Ruponen, E. Schacht, and A. Urtti. Structure-activity relationships of poly(L-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. *J. Control. Release*. **83**:169–182 (2002). doi:10.1016/S0168-3659(02)00178-5.
23. W. Li, Z. Huang, J. A. MacKay, S. Grube, and F. C. Szoka Jr. Low-pH-sensitive poly(ethylene glycol) (PEG)-stabilized plasmid nanolipoparticles: effects of PEG chain length, lipid composition and assembly conditions on gene delivery. *J. Gene Med.* **7**:67–79 (2005). doi:10.1002/jgm.634.
24. M. Oishi, Y. Nagasaki, K. Itaka, N. Nishiyama, and K. Kataoka. 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.* **127**:1624–1625 (2005). doi:10.1021/ja044941d.
25. G. F. Walker, C. Fella, J. Pelisek, J. Fahrmeir, S. Boeckle, M. Ogris, and E. Wagner. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer *in vitro* and *in vivo*. *Mol. Ther.* **11**:418–425 (2005). doi:10.1016/j.ymthe.2004.11.006.
26. A. A. Kale, and V. P. Torchilin. "Smart" drug carriers: PEGylated TATp-modified pH-sensitive liposomes. *J. Liposome Res.* **17**:197–203 (2007). doi:10.1080/08982100701525035.
27. V. Knorr, L. Allmendinger, G. F. Walker, F. F. Paintner, and E. Wagner. An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes. *Bioconjug. Chem.* **18**:1218–1225 (2007). doi:10.1021/bc060327a.
28. D. B. Rozema, D. L. Lewis, D. H. Wakefield, S. C. Wong, J. J. Klein, P. L. Roesch, S. L. Bertin, T. W. Reppen, Q. Chu, A. V. Blokhin, J. E. Hagstrom, and J. A. Wolff. Dynamic PolyConjugates for targeted *in vivo* delivery of siRNA to hepatocytes. *Proc. Natl. Acad. Sci U. S. A.* **104**:12982–12987 (2007). doi:10.1073/pnas.0703778104.
29. C. Fella, G. F. Walker, M. Ogris, and E. Wagner. Amine-reactive pyridylhydrazone-based PEG reagents for pH-reversible PEI polyplex shielding. *Eur. J. Pharm. Sci.* **34**:309–320 (2008). doi:10.1016/j.ejps.2008.05.004.
30. H. Hatakeyama, H. Akita, K. Kogure, M. Oishi, Y. Nagasaki, Y. Kihira, M. Ueno, H. Kobayashi, H. Kikuchi, and H. Harashima. Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. *Gene Ther.* **14**:68–77 (2007). doi:10.1038/sj.gt.3302843.
31. M. Meyer, and E. Wagner. pH-responsive shielding of non-viral gene vectors. *Expert. Opin. Drug Deliv.* **3**:563–571 (2006). doi:10.1517/17425247.3.5.563.
32. B. Brissault, A. Kichler, C. Guis, C. Leborgne, O. Danos, and H. Cheradame. Synthesis of linear polyethylenimine derivatives for DNA transfection. *Bioconjug. Chem.* **14**:581–587 (2003). doi:10.1021/bc0200529.
33. C. Plank, K. Zatloukal, M. Cotten, K. Mechtler, and E. Wagner. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjug. Chem.* **3**:533–539 (1992). doi:10.1021/bc00018a012.
34. F. Ungaro, G. De Rosa, A. Miro, and F. Quaglia. Spectrophotometric determination of polyethylenimine in the presence of an oligonucleotide for the characterization of controlled release formulations. *J. Pharm. Biomed. Anal.* **31**:143–149 (2003). doi:10.1016/S0731-7085(02)00571-X.
35. R. Kircheis, A. Kichler, G. Wallner, M. Kursa, M. Ogris, T. Felzmann, M. Buchberger, and E. Wagner. Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther.* **4**:409–418 (1997). doi:10.1038/sj.gt.3300418.
36. S. Boeckle, K. von Gersdorff, S. van der Piepen, C. Culmsee, E. Wagner, and M. Ogris. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J. Gene Med.* **6**:1102–1111 (2004). doi:10.1002/jgm.598.
37. K. von Gersdorff, M. Ogris, and E. Wagner. Cryoconserved shielded and EGF receptor targeted DNA polyplexes: cellular mechanisms. *Eur. J. Pharm. Biopharm.* **60**:279–285 (2005). doi:10.1016/j.ejpb.2004.08.014.
38. S. Han, R. I. Mahato, Y. K. Sung, and S. W. Kim. Development of biomaterials for gene therapy. *Mol. Ther.* **2**:302–317 (2000). doi:10.1006/mthe.2000.0142.
39. D. W. Pack, A. S. Hoffman, S. Pun, and P. S. Stayton. Design and development of polymers for gene delivery. *Nat. Rev. Drug Discov.* **4**:581–593 (2005). doi:10.1038/nrd1775.
40. E. Wagner, and J. Kloeckner. Gene delivery using polymer therapeutics. *Adv. Polym. Sci.* **192**:135–173 (2006). doi:10.1007/12_023.
41. D. Schaffert, and E. Wagner. Gene therapy progress and prospects: synthetic polymer-based systems. *Gene Ther.* **15**:1131–1138 (2008). doi:10.1038/gt.2008.105.
42. E. Wagner. Programmed drug delivery: nanosystems for tumor targeting. *Expert. Opin. Biol. Ther.* **7**:587–593 (2007). doi:10.1517/14712598.7.5.587.
43. J. A. Wolff, and D. B. Rozema. Breaking the Bonds: Non-viral Vectors Become Chemically Dynamic. *Mol. Ther.* **16**:8–15 (2008). doi:10.1038/sj.mt.6300326.