Dissertation

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Acetal containing polymers for the design of pH-responsive gene vectors

vorgelegt von

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Erklärung

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Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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1 Introduction

1.1 Stimuli-responsive drugs and drug delivery systems – towards programmed pharmaceutics

Efforts are currently made to advance traditional drugs or pharmaceuticals to more sophisticated and flexible systems. These are able to take into account specific environmental conditions in the patient and to respond to them in a desired way. Therefore, the drugs themselves or their pharmaceutical formulations are designed as programmed "stimuli-responsive" systems (*1-3*). This trend can be found in all areas of drug development and has already achieved success in many cases. The intentions thereby are multifarious and aim for example at the reduction of side effects and toxicity (on-site activation, *ciclesonide/Alvesco®* (*4*)(*5*)), the generation of specific kinetics in drug activation (pH-triggered activation of acid neutralization by *hydrotalcit (6,7)*), the protection of drugs from aggressive environments like gastric acid, or – the other way round – the protection of non-target sites in the patient from drug effects (enteric-coating of tablets or pellets, *Eudragit® (8)*), improvements in resorption (lipophilized prodrugs *Cefuroximaxetil, Oseltamivir (9, 10)*), et cetera. This concept of programmed stimuli responsiveness, already applied successfully for many conventional drugs and therapies, might also be advantageous in the field of biotechnological medicines, for instance in gene therapy.

1.2 Gene therapy – gene vectors

Many diseases are based on changes in the genome of the patient. Hence, a causal therapy tending not only to eliminate symptoms, but to cure a genetic disease, demands an intervention on the gene level. This means that the defective or missing gene has to be substituted in the cells of the concerned tissue. Examples for diseases that have already been cured with the help of gene therapy are cystic fibrososis (*11*) and severe combined immunodeficiency (SCID) (*12, 13*). But not only inherent genetic diseases can be treated by gene therapy; clinical trials have been performed also on the treatment of heart failure, other cardiovascular diseases, infectious diseases and more (*14*). Further, many cancers are susceptive to this kind

of treatment. The aim here is to either knock out genes that facilitate tumor growth, or to introduce therapeutic genes that – when expressed – antagonize tumor growth or cause apoptosis in tumor cells (p53, TNFα) *(15-17)(18)*. Gene therapy could offer a chance in cases where tumors are inaccessible to surgical removal, are drug resistant or have metastasized.

In the past, different methods have been developed to insert therapeutic genes into patient cells. The applied techniques can be distinguished into two main methods: *ex vivo* therapies, where affected cells are taken from the patient, transfected with the new gene outside the body and finally are re-implanted into the patient. In contrast, with the aid of gene vectors application of therapeutic genes can also be performed *in vivo*, which means that the therapeutic gene is directly administered to the patient. Vectors that are able to recognize their target cells need not be applied intratumorally, but can be injected systemically into the bloodstream. The vector then reaches its target cells via the circulation and gets internalized mostly by endocytosis. After subsequent release from the endosome into the cytoplasm, the therapeutic nucleic acid travels to the nucleus, where it has to be taken up; finally the transgene can be expressed. **Figure 1** shows schematically the steps of a vector based transfection process – additionally taking into account the barriers the vector has to overcome.

One possibility for vector mediated gene transfer is the use of viral vectors (*19*), which most commonly implies attenuated adeno- (*20*) or retroviruses. Thereby the viral genome is modified; undesired (i.e. useless, risky) parts are deleted and replaced by a therapeutic nucleic acid. The viral particles obtained retain their potential to infect cells, but cannot replicate any more. What makes these viral vectors so convenient is the fact, that viruses have constantly advanced their mechanisms of successfully attacking and infecting their hosts during evolution. Consequently, virus derived vectors profiting from this experience, convince by their high gene transfer efficiency. Viruses possess for instance mature strategies to overcome extra- and intracellular barriers (**Figure 1**) and to adapt themselves to changing conditions in the host, or even to utilize these for their own intentions.

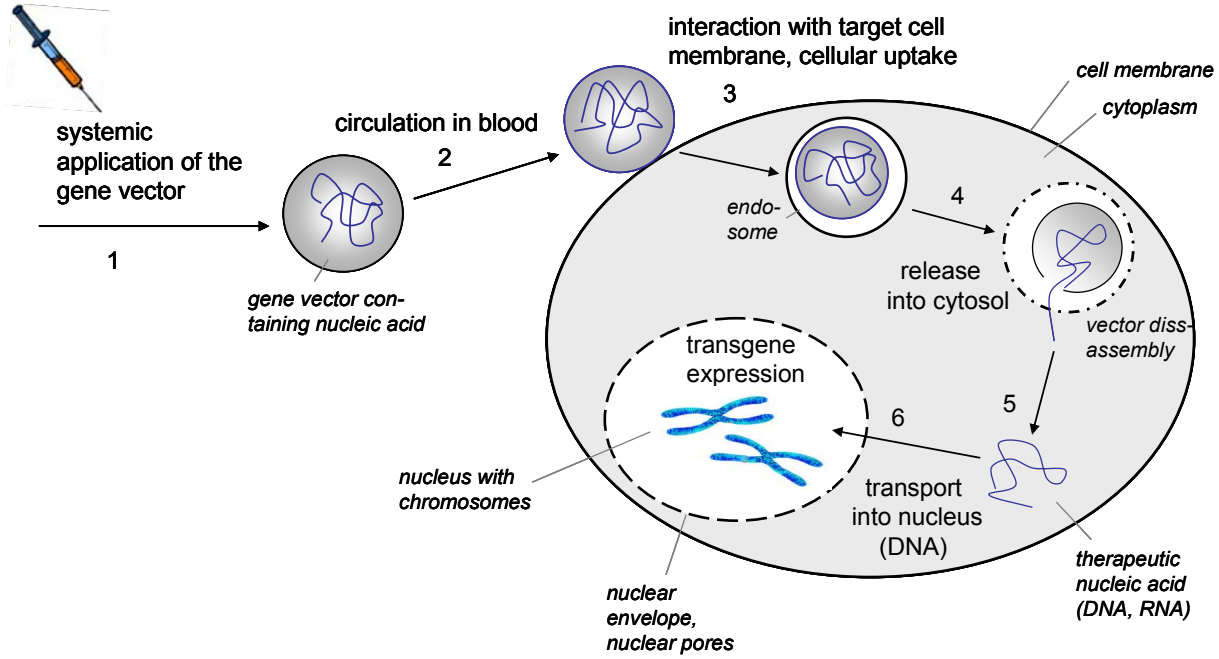


Figure 1. ***Route of a gene vector from the application site to the site of action. Extra- and intracellular barriers and hindrances the vector is confronted with:***
(1-2) Increased ionic strength in biological milieu favours aggregation; undesired interaction with blood components or non-target cells; inactivation by immune system/clearance by RES; degradation of nucleic acids by nucleases;
→ toxicity and/or inactivation of vectors
(3) Cellular uptake: cell membrane has to be overcome
(4) Escape into cytosol before recycling to cell surface or acidic/enzymatic degradation occur: passage through endosomal membrane;
(5) Cytosol as diffusion barrier; degradation of nucleic acid by cytosolic nucleases;
(6) Nuclear envelope has to be passed (in case of nuclear delivery/DNA)

For example receptors and other specific structures present on the target cell, are exploited for virus-binding and internalization into the cell (adenoviruses/fiber proteins (*20*)). Cytoskeletal structures and organelles in the cytoplasm represent a serious hindrance for the diffusion of macromolecules (*21, 22*) and therefore would impair cytoplasmic trafficking from vectors towards the nucleus. Viruses in contrast are able to take even advantage of this situation by utilizing the cellular structures (microtubules or actin microfilaments) as transport systems (*20, 21, 23*). Another aspect to mention are the sophisticated mechanisms viruses have developed to facilitate their release from endosome after cellular uptake by endocytosis (*24*). This includes for example the use of flexible viral structures that are able to respond to alterations in the environment by a change in their physicochemical properties.

Triggers therefore can be the contact with reductive milieu or a change in pH (*22, 23*), occurring for example between the endosomal compartment and extracellular or cytoplasmic milieu. Membrane fusiogenic residues for instance become activated upon acidification in the endosome (influenza virus: exposure of amphiphilic anionic peptide; adenovirus: hydrophobization of penton base protein) and induce endosomal release (*24*). These and further mechanisms make viruses so efficient in infecting and transfecting cells. However, the application of viral particles into a patient implicates not only advantages, but also holds several risks. Viral proteins can cause immunogenic reactions (*22, 25*), inappropriate insertion of genes into the host genome (insertional mutagenesis) can result in oncogenesis or other unpredictable diseases (*13*), and finally there still remains a residual risk that the altered virus regains its ability to generate infectious viral particles which cause diseases (*26*).

The alternative to viruses are non-viral delivery systems. This includes naked plasmid DNA (pDNA), as well as lipoplexes (composed of nucleic acids and cationic lipids), polyplexes (nucleic acids compacted by polycations) or combinations of these (*27, 28*). In contrast to naked DNA the use of cationic lipids and polycations has the advantage that anionic RNA or DNA macromolecules are compacted by these agents, which facilitates efficient cellular uptake and protection of the nucleic acids from nucleases. Compared to their viral counterparts non-viral vectors convince especially by the ease of synthesis, the lower costs in production, their improved immunogenic profile and better compatibility, and their high flexibility concerning the size of the transported gene construct (*29, 30*). However, these preferences are confronted with several insufficiencies that have to be compensated. Concerning the gene transfer efficiency, most non-viral vectors are still ranking far behind their viral counterparts (*22*). In addition, their toxicity profiles need to be improved further (*31, 32*). Thus the utilized polymers are often too large to be excreted (*33*) and most of them can not be degraded, as for example the widely used and efficiently transfecting polyethylenimines (PEI). Consequently, they accumulate in the patient´s organs and cause toxic side effects. However, substitution of the toxic high molecular weight polymers by well compatible small ones does not solve the problem, as along with polymer size also their transfection efficiency would get lost (*29, 34*).

Furthermore, many non-viral vectors – particularly polyplexes – exhibit a net positive surface charge due to the excess of polycation required for high gene transfer activity (*35*). During their circulation in the patient's blood, these positively charged particles can interact with negatively charged physiological compounds which results in significant toxicity and/or poor efficiency, due to binding to plasma proteins or blood cells, aggregation and complement activation (*36, 37*). Surface neutralization by PEGylation, i.e. the modification with the hydrophilic polymer poly(ethylene glycol) (PEG) prevents polyplexes from these undesired interactions as well as from rapid elimination by the reticuloendothelial system (RES) and thus decreases toxicity and extends circulation time (*38*). However, covering the polyplex surface with neutral polymers not only reduces undesired interactions, but also the desired binding to the negatively charged cell membrane of the target cells (*39*). Following the viral concept, the resulting reduced association and polyplex uptake into the cell can be overcome by the attachment of a targeting ligand to the polyplex for receptor-mediated uptake. PEI polyplexes containing PEG and cell-targeting ligands have been successfully applied *in vitro* and *in vivo* (*38-44*). While the uptake problem of PEG shielded particles can be solved satisfactorily, there still remain difficulties concerning the intracellular fate of PEGylated polyplexes. Extensive PEGylation, though being beneficial for systemic circulation, appears to negatively affect endosomal release of the delivered nucleic acid and leads to a remarkable reduction of transfection efficiency.

Thus, at the first view, it appears somehow contradictory to combine high transfection efficiency and low toxicity in just one vector. Indeed, non-viral vectors even when equipped with virus-like attributes like ligands are still not able to meet all the different requirements needed for successful transfection. What still distinguishes them from the flexible viruses is their static nature. Thus, if one would design non-viral vectors able to change their characteristics depending on the respective demands – i.e., to make them stimuli responsive like viruses – this inconsistency might be resolved.

1.3 Mimicking viruses` flexibility: pH-responsive non-viral gene vectors

Learning from nature – in particular from viruses – is the device for the design of more flexible, stimuli responsive and efficient non-viral gene delivery systems (*45, 46*). The ingenious mechanisms that viruses have developed (*23*) to overcome the barriers they are confronted with in their host (**Figure 1**) (*32*), can serve as guide towards the creation of improved non-viral vectors.

A polymer type equipped intrinsically with a virus like feature is for example polyethylenimine (i.e. PEI22K, PEI25K). High m.w. polyethylenimines mediate gene transfer very effectively. The special characteristic of these polycations is their particularly high density of protonable amines (*30, 47*), which act as stimuli responsive elements mediating endosomal release: when polyplexes are trapped in an endosome and the endosome matures to an endolysosome, the pH starts to drop. Upon this drop in pH PEI becomes further protonated. The increased positive polymer charge facilitates membrane interactions (*22, 32, 48*) and causes osmotic effects, leading to endosomal swelling and finally endosomal rupture (proton sponge hypothesis) (*22, 32, 49*). In this way PEI provokes the release of the vector into the cytoplasm before degradation in the endolysosome begins. This property of PEI reminds strongly of a viral attribute, as viruses also possess mechanisms for endosomal escape initiated by the acidification of the endosomal milieu (*22, 24*).

Nevertheless, PEI though often used as golden standard for non-viral transfections, still is not the perfect polymer. Inclusion of other stimuli triggered characteristics will be necessary to further improve vector characteristics and will be discussed below.

1.3.1 Acid-labile PEG shielding of gene vectors

As PEGylation improves polyplex characteristics in the extracellular compartment, but is impedimental for the following intracellular steps in the transfection process, it is obvious to think about methods of introducing PEG to polyplexes reversibly. Thus, when the PEG shield has become useless after cellular uptake of the polyplex, it will be deleted again. The cleavage of the linkage between the PEG and the polycation has to occur upon a specific stimulus present only in the endosome but not in the extracellular environment, to assure that polyplex deshielding does not occur before

endocytosis. Further, this cleavage has to take place fast enough to allow the subsequent endosomal release before transformation of the endosome into a lysosome, which would result in vector degradation. Following many viruses which use the pH drop occurring in the endosome as a trigger for their uncoating (*23*), non-viral vectors could also exploit this change in pH as stimulus for their deshielding.

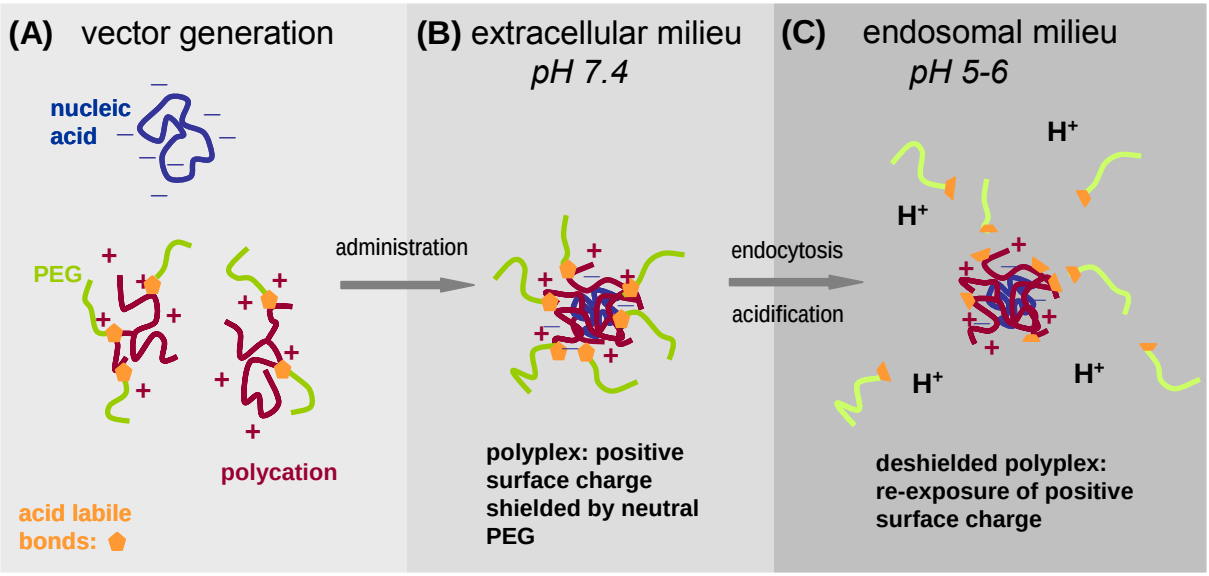


Figure 2. ***Schematic illustration of the concept of acid-sensitive PEGylation of polyplexes***
(A) Assembly of the vector out of nucleic acid and reversibly PEGylated polycation
(B) Systemic application of the gene vector to the patient; PEG shielding, stable in extracellular environment (physiologic pH of 7.4), prevents undesired interactions during circulation in the blood stream
(C) Upon endocytosis and endosomal acidification acid-labile linkages between PEG and polycation are cleaved and cause polyplex deshielding; recovery of positive surface charge facilitates polyplex release from endosome;

This implies that the PEG molecules have to be coupled to the vector via a pH-sensitive bond. Linker molecules that show an appropriate pH-sensitivity towards slight decreases in pH, such as the decrease from physiologic pH of 7.4 to endosomal pHs of about 6-5, can be found among hydrazones (*50*), vinyl ethers (*51*), orthoesters (*52-54*) and acetals (*55-57*). Lipoplexes and polyplexes equipped with such a pH-sensitive PEG shield have already been found to mediate enhanced gene transfer efficiency (*50, 52, 53*). Reversible shielding of PEI polyplexes for instance was achieved in an indirect manner by the conjugation of PEG to polylysine via a hydrazone bond (*50*). Unfortunately the chemistry was not applicable for direct

conjugation of PEG to PEI because of insufficient stability of the resulting PEI conjugate. Nevertheless, these data confirm that the concept of reversible PEGylation indeed is promising.

1.3.2 pH-sensitive degradable cationic carriers

PEGylation allows reducing toxicity of existing, prefabricated polycations like PEI or PLL retrospectively. Alternatively, if one does not rely on the commercial polymers, the aspect of an improved toxicity profile can already be considered when developing new polymer synthesis. Here, a particular interesting aspect is the design of polymers that can undergo biodegradation.

As mentioned above, high transfection efficiency is almost always associated with high vector toxicity (*31, 58*). For example polyethylenimines (PEI), macromolecular polycations of molecular weights around 22-25 kDa, mediate gene transfer very effectively but cause considerable toxic effects (*31*). Toxicity of PEIs is, among other things, based on the fact that these polymers are not degradable into small excretable degradation products. In contrast, shorter polyethylenimines such as 800 Da OEI possess just negligible toxicity, but exhibit only very poor gene transfer capacity. Therefore, one approach towards safer vectors is to design biodegradable polycations of adequate sizes, which allow gene transfer as effective as their stable PEI counterparts, but with time or upon a specific stimulus, decompose into non-toxic small degradation products. Several groups already have been working on this approach by inserting diverse degradable functions into their polymers. These functions include disulfide bonds, cleavable under reducing conditions (*58-62*); ester bonds, which hydrolyze upon time or can be cleaved by esterases (*33, 60, 63, 64*); pH-sensitive moieties as for example phosphoesters (*65, 66*), orthoesters (*67*), imines (*34*) or acetals/ketals (*68, 69*), disintegrating in acidic milieu. **Figure 3** schematically describes the concept of biodegradable gene vectors, exemplarily shown for an acid-sensitive polymer.

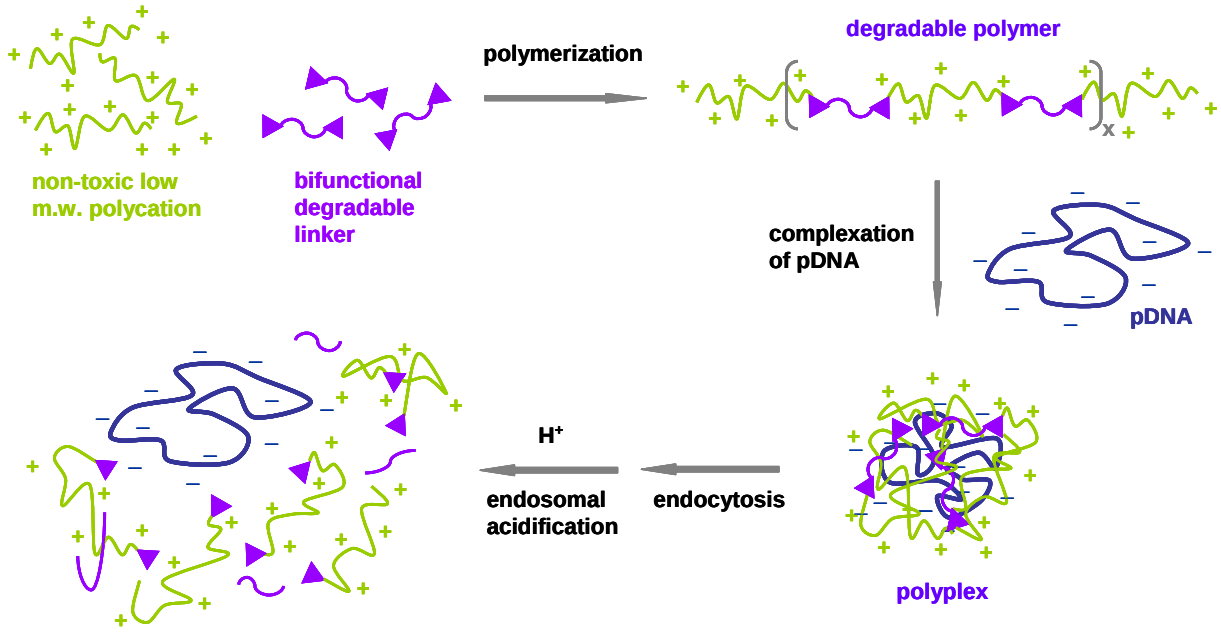


Figure 3. ***Generation of low-toxic, degradable gene vectors.*** *Low m.w. polycations are polymerized via bifunctional linker molecules containing predetermined breaking points. Self-assembly of the resulting degradable polymers with the therapeutic nucleic acid leads to condensed polyplexes of nanometric size, which protect the compacted DNA from nucleases and mediate endocytosis. Upon a specific stimulus – in case of acid-sensitive linkage the endosomal acidification – the cleavage of the breaking points is triggered, inducing polymer degradation into small low toxic units.*

1.4 Aims of the thesis

1.4.1 Synthesis and evaluation of a novel acetal-based PEGylation reagent for pH-sensitive shielding of polyplexes

As reversible PEGylation was shown to improve properties of non-viral gene vectors considerably, but no suitable universally applicable reagents for acid sensitive PEGylation of polycations were available so far, it was our intention to design such a versatile PEGylation reagent. In contrast to the PEG-hydrazone linker mentioned above (*50*) this new reagent should be compatible also with polycations like PEI, thus permitting the exclusion of PLL from the complexes. Further it should be

compatible with various targeting ligands, to allow efficient polyplex uptake into the cell despite the PEG shield.

The polymeric carriers Murthy et al. (*55, 56*) created by grafting hydrophilic PEG chains onto hydrophobic, membrane-disruptive methacrylate polymers, comprise acid-degradable *p*-amino-benzaldehyde acetals. These possess a suitable hydrolysis profile for hydrolysis at endosomal pH and therefore inspired us to choose as *p*-piperazino-benzaldehyde as staring point for our work. The aromatic aldehyde should be reacted with PEG alcohol to yield the corresponding acetal. Inclusion of a maleimido function (MAL) into the reagent should provide comfortable coupling of any desired thiol functionalized compound.

In a second step, the benefit of polyplexes reversibly PEGylated with the new maleimido-modified PEG acetal reagent (PEG-A-MAL) over analogous stably shielded ones should be investigated in biophysical and cell culture experiments.

1.4.2 Acetal linked, pH-degradable cationic carriers for reduced toxicity

The second intention of this work was the generation of new polycations for use as gene vectors with improved biocompatibility features. Therefore, small non-toxic but inefficient OEI800 units should be reacted to larger oligomers or polymers via acid degradable linkers. As a result of the increased size, these products should mediate gene transfer efficiently while still being non-toxic due to their endosomal degradability.

Acetal functions were chosen as predetermined breaking points, because of certain advantages: their acid-sensitivity makes them optimal agents for the targeting of endosomes as site of pH-induced cleavage. By adequate tailoring of their chemical properties, acetals allow to trigger polymer degradation exactly at a desired pH, such as the pH in endosomes. This would permit the production of vectors that are stabilized at physiological pH of 7.4 to mediate efficient transfer of nucleic acids from the circulation to the target cell. After endocytosis and endosomal acidification, when a high polymer m.w. is no longer needed, cleavage of the acid sensitive functions in the polymer would lead to vector degradation.

As such cationic polymers with pH-degradable acetal backbone had not been described before, the aim was the synthesis, characterization and application of two types of acetal linked OEI800 based polymers: MK-OEI polymers which include 2,2-

bis(*N*-maleimidoethyloxy) propane (MK) as pH-sensitive ketal linker (*70*), and BAA-OEI polymers wherein OEI moieties are linked via the bisacrylate acetal 1,1-bis (2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane) (BAA) (*71*). The advantage of the pH-sensitivity should be demonstrated by comparing the pH-labile polymers with homologous acid-stable polymers (BM-OEI and LT-OEI-HD), where the acid-sensitive functions are replaced by a stable ether or a hydrocarbon moiety, respectively. Finally, on the basis of MK-OEI polymers the influence of changes in biophysical properties on the toxicity/transfection efficiency profile should be evaluated.

2 Materials and methods

2.1 Chemicals and reagents

Toluene and tetrahydrofuran (THF) were distilled from sodium benzophenone ketyl radical, dimethyl sufoxide (DMSO) was distilled and stored over molecular sieves 4 Å under nitrogen. Acetone, dichloromethane (DCM), diethyl ether, ethyl acetate (EtOAc), chloroform, *n*-heptane, *n*-hexane, methanol and *N*-ethyldimethylamine were distilled prior to use. Diethyl ether was stored over KOH. Methoxy poly(ethylene glycol)maleimide (PEG-MAL, average molecular weight of 5 kDa) and methoxy poly(ethylene glycol) thiol, average m.w. 5 kDa (PEG-SH) were purchased from Nektar Therapeutics (Huntsville Alabama). Poly(ethylene glycol) 5 kDa monomethyl ether (mPEG) was purchased from Fluka Chemie GmbH (CH-9471 Buchs) and dried by heating to reflux (water separator) in toluene at an oil bath temperature of 170 °C for 20 h prior to use. 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 (*72*). Branched PEI of 1.8 kDa (PEI1.8K) was purchased from Polyscience, Inc. Warrington, USA. Poly-L-lysine, hydrobromide, with average m.w. of 48 kDa and of 58 kDa (PLL48K and PLL58K), oligoethylenimine with an average molecular mass of 800 Da (OEI800), branched PEIs with average molecular weights of 2, 10, 25 and 50 kDa (PEI2K, PEI10K, PEI25K, PEI50K), hexyl acrylate (HA), 1,6-hexanediol diacrylate (HD), *p*-methoxy-benzaldehyde, 2-hydroxyethyl acrylate, *p*-toluene sulfonic acid and triethylamine (TEA) were purchased from Sigma-Aldrich (Steinheim, Germany). Bafilomycin A1 was obtained from Alexis Biochemicals, CH-4415 Lausen, Switzerland; 1,8-Bis-maleimidodiethyleneglycol (BM) was purchased from Pierce Biotechnology, Inc., Rockford, USA, and 2,2-Bis(*N*-maleimidoethyloxy) propane (MK) from Organix Inc., Woburn, Massachusetts, USA. MK can be also synthesized as described by Srinivasachar et al. (*70*). 1,1-Bis-(2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane) (BAA) was synthesized following the protocol of Chan et al. (*71*). The resulting crude product was purified by flash column chromatography on silica gel equilibrated with the eluent (*n*-heptane/acetone 8.2/1.8 + 1 % TEA). BAA was obtained as a colorless oil with 100% purity as confirmed by ^{1}H NMR. Targeting conjugates (EGF-PEI and Tf-PEI) contain the ligands EGF or Tf linked with

branched PEI25K by a heterobifunctional 3.4 kDa PEG linker and were synthesized as described previously (*40, 41*). Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Fluka (Steinheim, Germany). Bis-(dibenzylideneacetone)palladium $Pd(dba)_2$ was obtained from Sigma-Aldrich (Steinheim, Germany). Tris-(dibenzylideneacetone)dipalladium ($Pd_2(dba)_3$) was a gift from Prof. Dr. Lorenz, LMU (Munich, Germany). 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*. (*73*) was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany).

General information on chemicals, methods and equipment

Water was used as purified, deionized water. All chemical procedures – except polymerization reactions – were carried out in oven-dried glassware under inert gas atmosphere (nitrogen or argon) unless stated otherwise. All reagents were purchased from commercial suppliers and used without further purification if not specified otherwise. To avoid stressing conditions during the polymerization reactions, all polymer syntheses were carried out under argon atmosphere in Eppendorff vials, in anhydrous DMSO. The reaction mixtures were kept at room temperature and were protected from light during the entire reaction time. Dialysis was performed with Spectra/Por membranes (molecular mass cut off 3.5 kDa or molecular mass cut off 6-8 kDa; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 4 °C. Infrared spectra were recorded on a Perkin Elmer FT-IR Spectrometer Paragon 1000 or on a Jasco FT/IR-410 spectrometer (Jasco Labor- und Datentechnik GmbH, Deutschland). ^{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 and refer to the solvent as internal standard (CH_2Cl_2 at 5.32 ppm, DMSO at 2.52 ppm, H_2O at 4.8 ppm). Data are reported as s = singulet, d = doublet, t = triplet, m = multiplet; coupling constants in Hz; integration. ^{13}C NMR spectra were recorded on a Jeol JNMR–GX500 (125 MHz) spectrometer. Chemical shifts are reported in ppm using solvent as an internal standard (CD_2Cl_2 at 5.38 ppm). Mass spectra were obtained on a Hewlett Packard 5989A MS Engine with 59980B Particle Beam and on a Jeol MStation JMS-700. UV absorption and UV spectra were recorded on a GENESYS^{TM} 10 Series Spectrometer (Thermo Electron

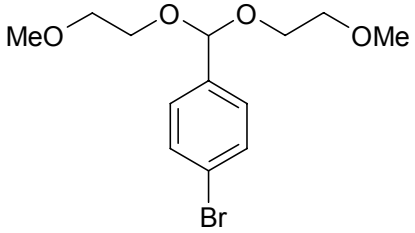
Corporation, Pittsford; NY). Column chromatography was performed on Merck silica gel 60 Å (40-63 µm). Gel filtration was performed on Sephadex G-25 superfine (20 mM Hepes buffer pH 7.4 with 0.25 M NaCl, at a flow rate of 1 mL/min), Superdex 75 prep grade or Superdex 200 prep grade (buffer as indicated respectively, at a flow rate of 1 mL/min) using a HR 10/30 column (Pharmacia, Sweden). Cation-exchange was performed on a HR 10/10 column (BioRad, Munich, Germany) filled with Macro-prep High S.

2.2 Design and synthesis of the acetal-based pH-sensitive PEGylation reagent PEG-A-MAL

2.2.1 Development of the synthesis on the basis of the dummy ME-A-MAL

1-[Bis-(2-methoxyethoxy)methyl]-4-bromobenzene (1). *p*-Bromobenzaldehyde (1.85 g, 10 mmol), *p*-TsOH·H_2O (19 mg, 0.1 mmol) and 2-methoxyethanol (1.66 ml, 21 mmol) were heated at 85 °C in $CHCl_3$ (50 ml) for 22 h by continuous removal of water. After cooling to r.t. a 20-fold excess of K_2CO_3 (0.2764 g, 2 mmol) was added and the reaction mixture was stirred for 1 h, then filtered and the solvent was removed under reduced pressure. The resulting residue was taken up in CH_2Cl_2, fixed onto silica gel and purified by column chromatography (*n*-hexane/EtOAc/CH_2Cl_2 = 9/0.5/0.5 + 1 % *N*-ethyldimethylamine) to afford **1** as yellow oil (1.41 g, 44 %).

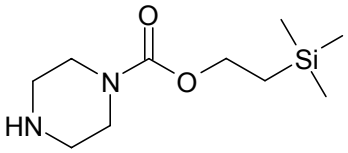
IR (film): 2979, 2925, 2877, 2818, 1592 cm^{-1}; ^1H NMR (500 MHz, CD_2Cl_2): δ = 3.33 (s, 6 H), 3.52 (t, *J* = 5 Hz, 4 H), 3.58-3.67 (m, 4 H), 5.55 (s, 1 H), 7.35-7.38 (m, 2 H), 7.48-7.51 (m, 2 H); ^{13}C NMR (125 MHz, CD_2Cl_2): δ = 59.0 (CH_3), 65.1 (CH_2), 72.2 (CH_2), 101.5 (CH), 122.7, 129.0, 131.6, 138.3 ($C_{arom.}$); MS (DEI^+): *m/z* (%) = 320 (1) [$M+H^+$], 59 (100).



1

2-Trimethylsilylethyl piperazine-1-carboxylate (2). To a solution of piperazine (260 mg, 3.0 mmol) in H_2O (3 ml) a solution of NEt_3 (0.632 ml, 4.5 mmol) in dioxane (3 ml) and a solution of 1-[2-(trimethylsilyl)ethoxycarbonyloxy]pyrrolidin-2,5-dion (39 mg, 1.5 mmol) in dioxane (3 ml) were added at r.t. subsequently. After stirring for 24 h the reaction mixture was treated with a saturated solution of $NaHCO_3$ (20 ml) and extracted with EtOAc (5 x 20 ml). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting residue was purified by column chromatography (EtOAc/CH_2Cl_2/MeOH = 5/4/2 + 1 % *N*-ethyldimethylamine) to afford **2** as colorless oil (211 mg, 61 %).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.02 (s, 9 H), 0.10 (t, *J* = 8.3 Hz, 2 H), 2.80-2.85 (m, 4 H), 3.42-3.48 (m, 4 H), 4.17 (t, *J* = 8.3 Hz, 2 H); MS (CI): *m/z* (%) = 231 (9) [M+H$^+$], 203 (100).

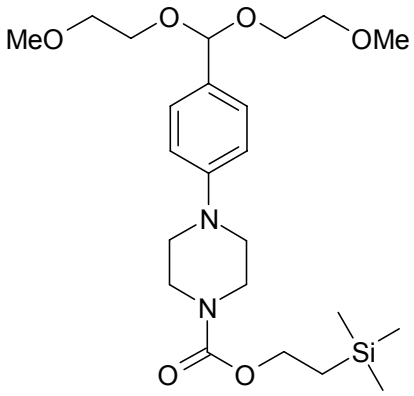


2

2-Trimethylsilylethyl 4-(4-{1-[bis(2-methoxyethoxy)]methyl}phenyl)piperazine-1-carboxylate (3). A solution of **2** (30.6 mg, 0.1328 mmol) and **1** (32.4 mg, 0.1014 mmol) in toluene (2 ml) was added to KO*t*Bu (17.1 mg, 0.1521 mmol), bis-(dibenzylideneacetone)palladium (5.8 mg, 0.0101mmol) and 2-(dicyclohexylphosphino)biphenyl (3.6 mg, 0.0101 mmol) and stirred at 90 °C for 18 h. After cooling to r.t. the mixture was treated with a saturated solution of $NaHCO_3$ (4 ml). The aqueous solution was extracted with CH_2Cl_2 (5 x 8 ml), the combined organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The resulting crude product was purified by column chromatography (equilibration with *n*-hexane/EtOAc/CH_2Cl_2 = 7/2/1 + 1 % *N*-ethyldimethylamine; eluent: *n*-hexane/EtOAc/CH_2Cl_2 = 7/2/1 + 0.5 % *N*-ethyldimethylamine) to afford **3** (38.0 mg, 80 %) as yellow oil .

IR (film): 2951, 2891, 2820, 1698, 1613 cm^{-1}; ^{1}H NMR (500 MHz, CD_2Cl_2): δ = 0.04 (s, 9 H), 1.01 (m, 2 H), 3.13 (t, *J* = 5.1 Hz, 4 H), 3.33 (s, 6 H), 3.49-3.67 (m, 12 H), 4.18 (m, 2 H), 5.49 (s, 1 H), 6.87-6.93 (m, 2 H), 7.30-7.36 (2 H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ = -1.4 (CH_3), 18.0 (CH_2), 44.0 (CH_2), 49.5 (CH_2), 59.0 (CH_3), 63.8 (CH_2),

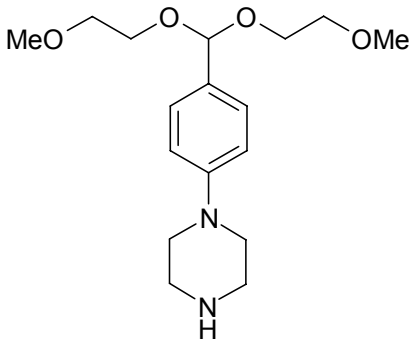
64.8 (CH_2), 72.2 (CH_2), 102.1 (CH), 116.1, 127.9, 130.4, 151.7 ($C_{arom.}$), 155.7 (C); MS (ESI^+): *m/z* (%) = 469 (53) [$M+H^+$], 393 (100), 491 (94) [$M+Na^+$]; UV (20 mM Hepes buffer pH 7.4/MeOH = 8/2): λ_{max} = 246 nm.



3

1-(4-{1-[Bis(2-methoxyethoxy)]methyl}phenyl)piperazine (4). A solution of TBAF·$3H_2O$ (30.2 mg, 0.0956 mmol) in THF (2 ml) was added dropwise to a stirred solution of **3** (22.4 mg, 0.0478 mmol) in THF (2 ml) at r.t.. After stirring for 24 h the reaction mixture was treated with a saturated solution of $NaHCO_3$ (5 ml), extracted with CH_2Cl_2 (5 x 5 ml) and the combined organic layers were dried ($MgSO_4$), filtered and the solvent was removed under reduced pressure. The resulting residue was purified by column chromatography (EtOAc/CH_2Cl_2 = 5/5 + 5 % *N*-ethyldimethylamine). The fractions containing **4** were contaminated with a salt derived from silicagel and *N*-ethyldimethylamine. For further purification they were evaporated and the resulting residue was extracted several times with EtOAc to separate the desired product from the EtOAc-insoluble salt. The combined EtOAc extracts were evaporated to afford **4** as a pale yellow oil (12.7 g, 82 %).

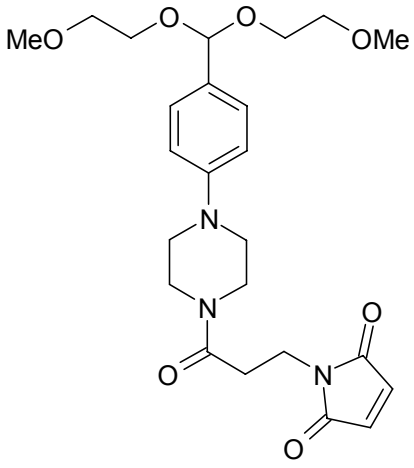
IR (film): 2925, 2879, 2820, 1613, 1518 cm^{-1}; ^1H NMR (500 MHz, CD_2Cl_2): δ = 2.66 (m, 2 H), 2.98-3.03 (m, 2 H), 3.12-3.21 (m, 4 H), 3.33 (s, 6 H), 3.50-3.66 (m, 8 H); ^{13}C NMR (100 MHz, CD_2Cl_2): δ = 46.2 (CH_2), 49.3 (CH_2), 50.2 (CH_2), 51.8 (CH_2), 59.9 (CH_3), 64.9 (CH_2), 72.2 (CH_2), 102.3 (CH), 115.4, 127.8, 129.5, 152.0 ($C_{arom.}$).



4

***N*-{3-[4-(4-{1-[bis-(2-methoxyethoxy)]methyl}phenyl)piperazinyl]-3- oxopropyl} maleimide (ME-A-MAL) (5).** To a stirred solution of **4** (8.6 mg, 0.0265 mmol) in DMSO (0.5 ml) *N*-methylmorpholine (3 µl, 0.0265 mmol) and 3-(maleimido)propionic acid *N*-hydroxysuccinimide ester (7.1 mg, 0.0265 mmol) were added subsequently at r.t.. After stirring for 2 h the reaction mixture was treated with phosphate buffer pH 7.4 [2 ml; KH_2PO_4 (250.0 ml, 0.2 M) + NaOH (393.4 ml, 0.1 M)] and extracted with EtOAc (4 x 4 ml). The combined organic layers were dried ($MgSO_4$), filtered and evaporated. The resulting crude product was purified by column chromatography (CH_2Cl_2/EtOAc = 7/3 + 1 % *N*-ethyldimethylamine) to afford **5** as yellow oil (6.9 mg; 55 %).

^{1}H NMR (500 MHz, CD_2Cl_2): δ = 2.64-2.73 (m, 2 H), 3.11-3.20 (m, 4 H), 3.33 (s, 6 H), 3.50-3.54 (m, 4 H), 3.54-3.66 (m, 8 H), 3.68-3.76 (m, 2 H), 3.78-3.84 (m, 2 H), 5.49 (s, 1 H), 6.69 (s, 2 H), 6.83-6.92 (m, 2 H), 7.31-7.36 (m, 2 H);



5

2.2.2 Synthesis of PEG-A-MAL

1-(4-Bromobenzene)-1-{bis[monomethoxypoly(ethylenoxy)]}methane (6). *p*-Bromobenzaldehyde (5.5506 g, 30 mmol) and *p*-TsOH·H_2O (19 mg, 0.1 mmol) were added to dried mPEG5K (10.0 g, 2 mmol) in toluene (50 ml), and the stirred reaction mixture was heated for 22 h at 170 °C (oil bath temperature). K_2CO_3 (276 mg, 2 mmol) was added and after 45 min of stirring at room temperature (r.t.) the solidified reaction mixture was diluted with with CH_2Cl_2, filtered and evaporated. For purification the crude product was powdered, washed with Et_2O (7 x) and finally dried under vacuum to afford **6** as a white powder (9.860 g, 97 %).
^{1}H NMR (500 MHz, CD_2Cl_2): δ = 3.32 (s, CH_3 PEG5K), 3.37-3.79 (m, CH_2 PEG5K), 5.56 (s, 1 H), 7.34-7.40 (m, 2 H), 7.45-7.52 (m, 2 H).

2-Trimethylsilylethyl 4-[4-(1{bis[monomethoxypoly(ethylenoxy)]}methyl)phenyl] piperazine-1-carboxylate (7). The following synthesis steps were carried out under an argon atmosphere. KO*t*Bu (19.1 mg, 0.17 mmol), $Pd_2(dba)_3$ (4.6 mg, 0.005 mmol), 2-(dicyclohexylphosphino)biphenyl and dried **6** (1.0167 g, 0.1 mmol) were suspended in toluene (1 ml). A solution of 2-trimethylsilylethyl piperazine-1-carboxylate (**2**, Supporting information) (34.5 mg, 0.15 mmol) in toluene (1 ml) was added and the reaction mixture was stirred for 45 h at 90 °C. After cooling to r.t. the mixture was treated with a saturated solution of $NaHCO_3$ (20 ml). The aqueous solution was extracted with CH_2Cl_2 (7 x 15 ml), the combined organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure. To remove impurities the resulting crude product was powdered and extracted with Et_2O (5 x) to give **7** as a fawn powder (918 mg, 90 %). The ^{1}H NMR shows this product contained about 82 % of **7** and 18 % of unreacted **6**, which could not be eliminated but also did not interfere in the following synthesis steps.
^{1}H NMR (500 MHz, CD_2Cl_2): δ = 0.04 (s, 9 H), 0.98-1.03 (m, 2 H), 3.13 (t, *J* = 5.0 Hz, 4 H), 3.32 (s, CH_3 PEG5K), 3.42-3.75 (m, CH_2 PEG5K, 4 H), 4.13-4.20 (m, 2 H), 5.50 (s, 1 H), 6.87-6.92 (m, 2 H), 7.31-7.36 (m, 2 H).

1-[4-(1-{Bis[monomethoxy poly(ethylenoxy)]}methyl)phenyl]piperazine (8). To a suspension of **7** (310 mg, 0.03 mmol) in THF (1 ml) a solution of tetrabutyl ammonium fluoride trihydrate (TBAF·3H_2O) in THF (1 ml) was added dropwise. After slightly warming the reaction mixture, it was stirred at r.t. for 20 h. The mixture was

then treated with a saturated solution of $NaHCO_3$ (5 ml). The aqueous solution was extracted with CH_2Cl_2 (5 x), the combined organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The resulting solid was powdered and washed with Et_2O (5 x 10 ml) to afford **8** as a beige powder (275.5 mg, 90 %).
^{1}H NMR (500 MHz, CD_2Cl_2): δ = 2.94-2.99 (m, 4 H), 3.07-3.12 (m, 4 H), 3.33 (s, CH_3 PEG5K), 3.42-3.76 (m, CH_2 PEG5K), 5.49 (s, 1 H), 6.84-6.90 (m, 2 H), 7.28-7.33 (m, 2 H).

***N*-(3-{4-[4-(1-{Bis[monomethoxy poly(ethylenoxy)]}methyl)phenyl] piperazinyl-} 3-oxopropyl) maleimide (9).** *N*-Methylmorpholin (11 µl, 0.1 mmol) and *N*-succinimidyl-3-maleimidopropionate (13 mg, 0.05 mmol) were added subsequently to a suspension of 8 (101.9 mg, 0.01 mmol) in DMSO (0.5 ml) and stirred at r.t. for 30 min. The reaction mixture was then treated with phosphate buffer pH 7.4 [3 ml; KH_2PO_4 (250.0 ml, 0.2 M) + NaOH (393.4 ml, 0.1 M)] and extracted with CH_2Cl_2 (5 x 5 ml). The combined organic layers were dried ($MgSO_4$), filtered and evaporated. The resulting beige solid was aliquoted into two portions of about 50 mg, dissolved in 20 mM Hepes buffer pH 7.4 with 0.25 M NaCl (2 ml) and purified by gel filtration chromatography (G-25 superfine). The fractions containing the desired product were extracted with CH_2Cl_2 (5 x 5 ml). The combined organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure to give **9** as a pale yellow solid (73.5 mg, 71 %).
^{1}H NMR (500 MHz, CD_2Cl_2): δ = 2.63-2.70 (m, 2 H), 3.11-3.20 (m, 2 H), 3.32 (s, 6 H), 3.42-3.82 (m, CH_2 PEG5K, 4H, 4 H), 5.50 (s, 1 H), 6.69 (s, 2 H), 6.86-6.91 (m, 2 H), 7.32-7.36 (m, 2 H).

2.3 PEGylation of polycations

2.3.1 Thiolation

Thiolation of branched polyethylenimine. PEI25K (6 mg, 0.24 µmol) in 0.1 M Hepes buffer pH 7.4 (0.4 ml) was mixed with 0.6 µmol SPDP from a stock at 3 mg/mL in DMSO. The reaction was mixed over night at room temperature and the

resulting product was purified by G-25 superfine gel filtration (*74, 75*). The dithiopyridyl groups of the product were reduced with 50 mol equivalents of DTT for 10 min and the resulting thiol modified PEI25K was purified by G-25 superfine gel filtration equilibrated in 20mM Hepes pH 7.4 containing 0.25 M NaCl under argon. The product was snap frozen in liquid nitrogen and stored at -80 °C. PEI25K was quantified by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay (*76*) and the amount of free mercapto groups was determined by the Ellman`s assay (*77*).

Thiolation of poly-(L-lysine) (PLL58K), linear polyethylenimine (PEI22K), HT-OEI-HD1. PLL58K, PEI22K and HT-OEI-HD1 were thiolated in analog manner to PEI25K. Quantification of polycation in the product was performed by TNBS assay, thiol groups were quantified by Ellman´s assay.

2.3.2 PEGylation: poly(ethylene glycol)-polycation conjugates

Poly(ethylene glycol)-polyethylenimine conjugates. PEG-A-MAL **9** (0.2 ml, 0.12 µmol) for acid reversible conjugates, or PEG-MAL (0.1 ml, 0.2 µmol) for acid-stable conjugates were dissolved in degassed 20 mM Hepes buffer pH 7.4 containing 0.25 M NaCl by brief sonication. PEG-A-MAL or PEG-MAL was mixed with PEI25K-SH at 0.03 or 0.02 µmol (PEI), respectively (total volume 2 ml). After 10 min the reaction was applied to the cation-exchange column which was equilibrated in 20 mM Hepes buffer pH 7.4 containing 0.5 M NaCl. The salt concentration was increased with a linear gradient to 3 M NaCl (10 min) and maintained at 3 M NaCl for 15 min with a flow rate of 1 ml/min. The PEI25K product was eluted as a single peak at 3M NaCl. PEI25K was quantified by the TNBS assay. PEG-conjugation was monitored by measuring unbound PEG (*78*) and by ^{1}H NMR. The purified conjugates (PEG-A-PEI25K and PEG-S-PEI25K) were snap frozen in liquid nitrogen and stored at -80 °C.

PEGylation of PLL58K, PEI22K, HT-OEI-HD1. For acid reversible PEGylation the thiolated polycations were reacted with PEG-A-MAL **9** as described above for PEI25K-SH.

2.4 Synthesis of acetal linked acid-degradable MK polymers, stable BM control polymers, and their derivatives

2.4.1 Synthesis of pH-stable dummy polymer BM-A1/1

BM (15.4 mg; 50 mg/mL in DMSO) was added dropwise and under vortexing to OEI800 (40 mg; 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 1M Hepes buffer pH 7.5 containing 4 M sodium chloride. 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 exchanged against water; water was then replaced twice by fresh water. After a total dialysis duration of 26 h the purified product was lyophilized (yield 48 %) and stored at -80 °C. According to ^{1}H NMR analysis, the molar ratio of BM to OEI800 in the product was 1.3/1.

^{1}H NMR (500 MHz, D_2O): δ = 3.77 (t, 4H, NC*H*$_2$CH_2O), 3.69 (s, 4H, OC*H*$_2$C*H*$_2$O linker ethylene), 3.64 (t, 2H, COC*H*CH_2 of linker succinimide), 3.59-2.35 (br m; 72H NC*H*$_2$ of OEI ethylenes; 4H NCH_2C*H*$_2$O of linker ethylenes and 4H COC*H*$_2$CH of linker succinimide).

2.4.2 Synthesis of pH-stable dummy polymer BM-B1/1

The polymer was synthesized in an analog manner as BM-A1/1. Therefore, BM (15.4 mg; 50 mg/mL in DMSO) was added dropwise and under vortexing to PEI1.8K (90 mg; 600 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 4M sodium chloride and dialyzed at 4 °C using a Spectra/Por membrane (molecular mass cut off 6-8 kDa) for 26 h as described above. After dialysis, the purified product was lyophilized and stored at -80 °C (yield 68 %). According to ^{1}H NMR analysis, the molar ratio of BM to PEI1.8K in the product was 1.3/1.

^{1}H NMR (400 MHz, D_2O): δ = 3.72 (t, 4H, NC*H*$_2$CH_2O), 3.69-3.54 (m, 4H, OC*H*$_2$C*H*$_2$O linker ethylene; 2H, COC*H*CH_2 of linker succinimide), 3.54-2.30 (br m;

162H, NCH_2 of PEI ethylenes; 4H NCH_2CH_2O of linker ethylenes and 4H $COCH_2CH$ of linker succinimide).

2.4.3 Synthesis of pH-stable dummy polymer PEG-BM-A1/1

A solution of PEG-SH (14.2 mg; 333 mg/mL in DCM) was added dropwise and under vortexing to a 19-fold excess of BM (15.4 mg; 50 mg/mL in DMSO). After mixing the components thoroughly, the solution was added dropwise and under vortexing to OEI800 (38 mg; 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 %). According to ^{1}H NMR analysis, the molar ratio of PEG to BM to OEI800 in the product was 0.05/1.3/1;

^{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).

2.4.4 Synthesis of acid-degradable polymers MK-A1/1, MK-A1.2/1, MK-A1.5/1

MK (16.1 mg, 19.32 mg, or 24.15 mg respectively; 50 mg/mL in DMSO) was added dropwise and under vortexing to OEI800 (40 mg; 400 mg/mL in DMSO) and reacted for 22 h at room temperature while shaking constantly. Quenching, purification and lyophilization were performed as indicated above for BM-A1/1, unless for dialysis of MK-A1.2/1 and 1.5/1, where the 3.5 kDa Spectra/Por membrane was exchanged by a 6-8 kDa cut off membrane. Dry polymers were stored at -80 °C (yields were 40 % for MK-A1/1, 51% for MK-A1.2/1 and 60 % for MK-A1.5/1 polymer). According to ^{1}H NMR analysis molar ratios of MK linker acetals versus OEI800 in the product were 0.93/1 (MK-A1/1), 1.05/1 (MK-A1.2/1) and 1.23/1 (MK-A1.5/1).

^{1}H NMR (400 MHz, D_2O): δ = 3.75 (t, 4H, OC*H*$_2$), 3.72-2.30 (br m; 72H NC*H*$_2$ of OEI ethylenes; 10H NC*H*$_2$ of linker ethylene and COC*H*C*H*$_2$ of linker succinimide), 1.45-1.25 (m, 6H, C*H*$_3$ of linker acetone ketal).

2.4.5 Synthesis of acid-degradable MK-B1/1

MK (16.1; 50 mg/mL in DMSO) was added dropwise and under vortexing to PEI1.8K (90 mg; 600 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 and dialyzed at 4 °C using a Spectra/Por membrane (molecular mass cut off 6-8 kDa) for 26 h as described above. After a total dialysis duration of 26 h, the purified product was lyophilized and stored at -80 °C (yield 43 %). According to ^{1}H NMR analysis, the molar ratio of MK linker acetals to PEI1.8K in the product was 0.93/1.

^{1}H NMR (400 MHz, D_2O): δ = 3.77 (t, 4H, OC*H*$_2$), 3.81-2.40 (br m; 162H NC*H*$_2$ of PEI ethylenes; 14H NC*H*$_2$C*H*$_2$O of linker ethylene and COC*H*C*H*$_2$ of linker succinimide), 1.45-1.24 (m, 6H, C*H*$_3$ linker).

2.4.6 Synthesis of acid-degradable PEG-MK-A1/1

A solution of PEG-SH (14.2 mg; 333 mg/mL in DCM) was added dropwise and under vortexing to MK (16.0 mg; 50 mg/mL in DMSO). After mixing the components thoroughly, the solution was added dropwise and under vortexing to OEI800 (38 mg; 400 mg/mL in DMSO) and reacted for 22 h at room temperature under constant shaking. Quenching, purification and lyophilization were performed as indicated above for PEG-BM-A1/1 (yield 43 %). According to ^{1}H NMR analysis, the molar ratio of PEG to MK to OEI800 in the product was 0.08/1.09/1.

^{1}H NMR (400 MHz, D_2O): δ = 3.86-2.30 (br m; 472H OC*H*$_2$C*H*$_2$O of PEG ethylene; 72H NC*H*$_2$ of OEI ethylenes; 14H NC*H*$_2$C*H*$_2$O of linker ethylene and COC*H*C*H*$_2$ of linker succinimide), 1.45-1.25 (m, 6H, C*H*$_3$ linker).

2.4.7 Synthesis of acid-degradable HA2.5-MK-A1/1, HA5-MK-A1/1, HA10-MK-A1/1

MK-A1/1 was synthesized following the protocol above. After the reaction time of 22 h a 2.5-, 5- or 10-fold molar amount (related to molar amount of OEI800) of hexyl-acrylate (19.5mg, 22.0 µl; 39.1 mg, 44.0 µl; or 78.1mg, 88.0 µl respectively) was added and the mixture was kept on the shaker at 40 °C for another 3 h. Prior to purification, the reaction mixture was diluted 1:4 with 1 M Hepes buffer pH 7.5 containing 4 M sodium chloride and dialyzed at 4 °C using a Spectra/Por membrane (molecular mass cut off 3.5 kDa) for 26 h as described above. After total dialysis duration of 26 h, the purified product was lyophilized and stored at -80 °C (yield 66 %, 70 % and 84 % for HA2.5-, HA5- and HA10-MK-A1/1 respectively). According to ^{1}H NMR analysis, the molar ratios of HA/MK/OEI in the products were 1.4/0.93/1 (HA2.5-MK-A1/1), 3.6/0.93/1 (HA5-MK-A1/1) and 7.0/0.93/1 (HA5-MK-A1/1).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 4.08-3.96 (m; 2H COOCH_2 HA-ester), 3.53 (s; 472H OCH_2CH_2O of PEG ethylene), 3.49-2.20 (m; 72H NCH_2 of OEI ethylenes; 14H NCH_2CH_2O of linker ethylene and COCHCH_2 of linker succinimide; 4H NCH_2CH_2COO of HA), 1.64-1.45 (m; 2H COOCH$_2$CH_2 HA-ester); 1.39-1.12 (m; 6H CH_2CH_2CH_2CH$_3$ of HA; 6H, CH_3 linker), 0.96-0.76 (m; 3H CH_3 HA);

2.4.8 Synthesis of acid-degradable HA2.5-PEG-MK-A1/1, HA5-PEG-MK-A1/1, HA10-PEG-MK-A1/1

PEG-MK-A1/1 was synthesized following the protocol above. After the reaction time of 22 h a 2.5-, 5- or 10-fold molar amount (related to molar amount of OEI800) of hexyl acrylate (18.6mg, 20.9 µl; 37.1 mg, 41.8 µl; or 74.2mg, 83.6 µl respectively) was added and the mixture was kept on the shaker at 40 °C for another 3 h. Prior to purification, the reaction mixture was diluted 1:4 with 1 M Hepes buffer pH 7.5 containing 4 M sodium chloride and dialyzed at 4 °C using a Spectra/Por membrane (molecular mass cut off 3.5 kDa) for 26 h as described above. After total dialysis duration of 26 h, the purified product was lyophilized and stored at -80 °C (yield 62 %, 74 % and 47 % for HA2.5-, HA5- and HA10-PEG-MK-A1/1 respectively). According to ^{1}H NMR analysis, the molar ratios of HA/PEG/MK/OEI in the products were 1.3/0.084/1.09/1 (HA2.5-PEG-MK-A1/1), 3.6/0.047/1.09/1 (HA5-PEG-MK-A1/1) and 7.4/0.041/1.09/1 (HA5-PEG-MK-A1/1).

^{1}H NMR (500 MHz, DMSO-d_6) δ = 4.05-3.96 (m; 2H COOCH_2 HA-ester), 3.50-2.11 (m; 72H NCH_2 of OEI ethylenes; 14H NCH_2CH_2O of linker ethylene and COCHCH_2 of linker succinimide; 4H NCH_2CH_2COO of HA), 1.61-1.52 (m; 2H COOCH$_2$CH_2 HA-ester); 1.37-1.16 (m; 6H CH_2CH_2CH_2CH$_3$ of HA; 6H, CH_3 linker), 0.92-0.83 (m; 3H CH_3 HA).

2.5 Synthesis of ketal linked acid-degradable BAA polymers

2.5.1 Synthesis of acid-degradable BAA-1/1-3d, -1.5d, -45min, BAA-2/1-3d and BAA-1.25/1-45min-hc

BAA (14 mg (for synthesis of BAA-1/1 polymers) and 28 mg (for synthesis of BAA-2/1), each 50 mg/mL in DMSO; or 17.5 mg (for synthesis of BAA-1/1-45min-hc), 500 mg/mL in DMSO) was added dropwise and under vortexing to OEI800 (32 mg; 400 mg/mL in DMSO) and reacted for 3 days (BAA-1/1-3d, BAA-2/1-3d), 1.5 days (BAA-1/1-1.5d) or 45 min (BAA-1/1-45min, BAA-1.25/1-45min-hc) at room temperature with constant shaking. Quenching, purification (total dialysis duration was only 15 h to prevent high extents of aminolysis) and lyophilization were performed as indicated above for BM-A1/1. The yields were 40 %, 56 % and 70 % in case of BAA-1/1-3d, -1.5d and -45min, respectively; 50 % yield in case of BAA-2/1-3d and 58 % in case of BAA-1.25/1-45min-hc. Polymers were stored at -80 °C. According to ^{1}H NMR analysis the molar ratios of BAA linker versus OEI800 in the products were 0.3/1 (BAA-1/1-3d) and 0.7/1 (BAA-2/1-3d), the content of intact diester therein was 26 % for BAA-1/1-3d and BAA-2/1-3d; if reaction time was shortened to 1.5 days (BAA-1/1-1.5d), the BAA/OEI ratio was 0.4 with a diester content of 40 %. Reduction of the reaction time to 45 min increased the ratio to 0.44 with 75 % diester (BAA-1/1-45min) and 0.56 with 60 % diester (BAA-1.25/1-45min-hc). ^{1}H NMR spectra of the purified polymers, which were recorded in D_2O, gave the following identification:

^{1}H NMR (400 MHz, D_2O): δ = 7.50-7.37 (m, 2H, CH CHCOCH$_3$ of aromatic ring), 7.08-6.96 (m, 2H, CH COCH$_3$ of aromatic ring), 5.68-5.53 (m; 1H, acetal H), 4.35-4.28 (m, COOCH_2; 2H, monoester* or 4H, diester), 4.00-2.30 (br m; 72H NCH_2 of OEI ethylenes; 3H, OCH$_3$; 4H, OCH_2CH$_2$OCO; 2H, OCH$_2$CH_2OH*; 8H, OOCCH_2CH_2

and NOCCH_2CH_2*). *Due to partial ester hydrolysis and aminolysis, resulting in corresponding alcohol and the carboxylic acid or amide.

2.5.2 Synthesis of LT-OEI-HD polymer

Synthesis of LT-OEI-HD was performed following the protocol of Kloeckner et al. (*79*). HD-linker and OEI800 were employed in the synthesis at a molar ratio of 1.2/1 (HD/OEI800).

2.6 Polymer characterization methods

2.6.1 Trinitrobenzenesulfonic acid (TNBS) assay

Concentration of PLL, PEI, PEI conjugates and BAA polymers was measured by trinitrobenzenesulfonic acid (TNBS) assay as described in (*76*). In a 96-well plate standard solutions of PLL, PEI or OEI800, respectively, with a defined polymer concentration, and test solutions containing the samples were serially diluted in duplicates with 0.1 M sodium tetraborate to give a final volume of 100 µl. Applied standard polymer concentrations ranged from 0 to 60 µg/ml, in steps of 10 µg/ml. 2.5 µl of TNBS (75 nmol) diluted in water was added to each well. After a reaction time of 5 minutes at room temperature, absorption was measured at 405 nm using a microplate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria).

2.6.2 Copper complex assay

Quantification of OEI800- or PEI1.8K-based polymers (except BAA-polymers) was performed by a copper complex assay described in (*80*): 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/PEI1.8K dilutions of known concentrations (standard curve) or with 50 µl of the OEI/PEI1.8K-based polymer solutions. The resulting Cu(II)/amine complexes were quantified by measuring the absorbance at 285 nm

using a GENESYS^{TM} 10 Series Spectrometer (Thermo Electron Corporation, Pittsford, NY).

2.6.3 Analysis of aminolysis kinetics by ^{1}H NMR

Synthesis of BAA-1/1 was performed as indicated above but by exchanging DMSO by DMSO-d_6. The reaction mixture was analyzed by ^{1}H NMR at different time points between 0.5 h and 24 d. The degree of amininolysis was calculated by analyzing the signal of the acetal proton at 5.5 ppm, which shows a shift towards the higher field when aminolysis occurs.

2.6.4 Size exclusion chromatography (SEC) for determination of polymer molecular weight and for polymer fractionation

Gel filtration was performed on Superdex 75 or a Superdex 200 material (GE Healthcare Bio Sciences AB, SE-75184 Uppsala) using a HR 10/30 column (Pharmacia, Sweden) 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 to saturate residual ionic functions, which might interact with amine containing samples and impair the results. 20 mM Hepes buffer pH 7.4 with 0.25 M NaCl was chosen as running buffer. Samples were dissolved in the running buffer and applied to the column filled with either Superdex 75 material for determinations in the low molecular weight or Superdex 200 for the analysis in the high molecular weight range. Runs were performed at a flow rate of 1 mL/min and sample detection was carried out at λ = 225 nm and 254 nm.

Polymer fractionation (MK-A1/1, MK-B1/1) was performed analogously using a Superdex 75 column and 20 mM Hepes buffer pH 7.8 with 0.25 M NaCl as solvent and running buffer. The polymers were separated into three fractions, starting with Fr.I which comprises the polymer-portion eluting between minute 9 and 15, followed by Fr.II ranging from minute 16 to 20 and finally closing with Fr.III, containing monomers eluting after 21 minutes and later.

For determination of the acid degradability of BAA polymers, the polymers were diluted in 20 mM Hepes and adjusted at pH 1 by the addition of HCl. Samples were incubated at 37 °C for 1 h, finally neutralized with NaOH and analyzed for size on a Superdex 75 column with 20 mM Hepes pH 7.4, 0.25 M NaCl as running buffer.

2.6.5 Determination of polymer molecular weight by gel permeation chromatography (GPC)

Additional analysis of acid-stable polymers (BM-A1/1, BM-B1/1, LT-OEI-HD) was performed using an Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a refractive index detector, a Novema 10-µm precolumn and a Novema 300 analytical column (10 µm, 8 x 300 mm) (PSS, Mainz, Germany). The mobile phase was maintained in formic acid and sodium chloride (0.1 % (v/v) HCOOH, 0.1 M NaCl, pH 2.8) at a flow rate of 1 mL/min. Samples were dissolved in the mobile phase at a concentration of 6 mg/mL; 10 µl of methanol were added to 1 mL of sample as internal standard. Results were evaluated using PSS WinGPC Unity software. Molecular weights were measured relative to Pullulan molecular weight standards used for preparing a standard calibration curve.

2.6.6 Hydrolysis assays

Hyrolysis kinetics of the acetal linkage in PEG-A-PEI25K

The PEG-A-PEI25K conjugate (0.0092 µmol, 0.8 ml) was adjusted to pH 5 by the addition of sodium acetate (0.333 M) to a final concentration of 0.2 M total volume 2 ml and was incubated for 22 h at 37 °C. The reaction was applied to and separated on the cation-exchange column and the unbound PEG fractions were pooled and the amount of PEG was quantified (*78*).

For hydrolysis kinetics measurements compound 9 was dissolved in the respective buffer (0.2 M Hepes buffer pH 7.4; 0.2 M Hepes buffer pH 7.0; 0.2 M phosphate buffer pH 6.5; 0.2 M phosphate buffer pH 6.0; 0.2 M phosphate buffer pH 5.5; 0.5 M NaOAc buffer pH 5.0) at a concentration of 1.5 mg/ml.

Hyrolysis kinetics of MK and BM linked polymers

Aliquots of MK-A1/1 or BM-A1/1 were dissolved either in 1 mL 20 mM Hepes buffer pH 7.4 containing 0.25 M NaCl or in 1 mL 0.2 M sodium acetate buffer pH 5.0 containing 0.25 M NaCl. After incubation at 37 °C for different time periods, samples were applied to a gel filtration column (Superdex 75) either directly (pH 7.4 samples) or after neutralization with sodium hydroxide to stop hydrolysis (pH 5.0 samples). SEC was performed as indicated above. For calculation of the degradation rates, the

monitored curves were segmented, integrated and the integrals were set into relation.

Hyrolysis kinetics of BAA linked polymers

For BAA polymer kinetics of acetal hydrolysis was measured by monitoring the UV absorption of the emerging hydrolysis product (*p*-methoxy-benzaldehyde) at 300 nm. Therefore, the polymer was diluted with 0.2 M sodium acetate buffer pH 5.0 for the pH 5.0 measurements, while 0.2 M Hepes buffer pH 7.4 was used for the pH 7.4 kinetics. Samples were incubated at 37 °C and absorption was determined on a GENESYS^{TM} 10 Series Spectrometer (Thermo Electron Corporation, Pittsford, NY) at different time points.

Hyrolysis kinetics of HD linked polymer

Hydrolysis of LT-OEI-HD was monitored by ^{1}H NMR analysis as the polymer lacks appropriate UV absorbing functions. Therefore the polymer was incubated in D_2O at pH 7.4 and 37 °C. After different periods of time – up to 14 days – ^{1}H NMR spectra were recorded and the decrease of the ester signal was analyzed.

2.6.7 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.

2.6.8 Formation of polyplexes

Plasmid DNA (pDNA) was mixed with the polycation at the indicated ratios. In case of PEI polyplexes these ratios are given as molar ratios of PEI nitrogen atoms to DNA phosphate (N/P); various PEI derivatives were included in the formulation at the indicated ratios, as unmodified PEI (PEI22K or PEI25K), targeting conjugate (EGF-PEI or Tf-PEI) or shielding conjugate (PEG-S-PEI or PEG-A-PEI). In case of OEI800- or PEI1.8K-based polymers the ratios are given as cation/pDNA (c/p) ratios (w/w). For PEI, c/p 0.78 presents N/P 6. Polycation/DNA polyplexes were prepared as described in (*75*) 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 polycations were each diluted in HBG or HBS pH 7.4 and mixed rapidly by pipetting. Polyplexes were incubated at room temperature for 20 min prior to use.

2.6.9 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 x 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 polyplexes dilutions were incubated at 37 °C for the indicated time periods.

2.6.10 Cell culture

Renca-EGFR mouse renal carcinoma cells stably transfected with pLTR-EGFR and pSV2neo (kindly provided by Winfried Wels, Frankfurt am Main, Germany) and K562 suspension cells (ATCC CCL-243) were grown in RPMI-1640 with 4 % Glutamax I medium supplemented with 10 % FCS and 1 % antibiotics (streptomycin, penicillin). B16F10 murine melanoma cells (kindly provided by I.J. Fidler, Texas Medical Center, Houston, TX) and murine neuroblastoma Neuro2A cells (ATCC CCI-131) were cultured in DMEM (1 g of glucose/L) supplemented with 10 % FCS (v/v) and 1 % penicillin/streptomycin (v/v). All cells were grown at 37 °C in 5 % CO_2 humidified atmosphere.

2.6.11 Luciferase reporter gene expression and metabolic activity of transfected cells

Transfection experiments concerning the acid-labile PEG shielding of EGF- or Tf-receptor targeted PEI polyplexes were performed on Renca-EGFR or K562 cells. Renca-EGFR cells were seeded in 96-well plates with 5 x 10^3 cells per well 24 h

prior to transfection. K562 (2.5 x 10^5 cells in 0.75 mL) were plated in 24-well plates; Transfection polyplexes were added to each well at the amounts of 200 ng pCMVL plasmid DNA per well in case of Renca-EGFR cells and 2.5 µg in case of K256 cells. After 4 h of incubation at 37 °C the transfection medium was replaced by fresh culture medium. Luciferase gene expression was measured after 24 h as described (*81*). Values are given as relative light units (RLU) and represent the luciferase activity per 10^4 (Renca) or 2.5 x 10^5 (K562) cells. 2 ng of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^7 light units.

In vitro transfection experiments with the OEI800- and PEI1.8K-based polymers were performed on either B16F10 or Neuro2A cells. Two parallel transfection series were carried out in separate 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 in 96-well plates with a density of 5 × 10^3 (B16F10) or 1 × 10^5 cells (Neuro2A) 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. In case of experiments with bafilomycin A1, bafilomycin A1 (1 mM in DMSO) was added to the complex dilutions to give a final concentration of 100 nM, before addition 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 (*35*). 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 as described in (*82*). 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 %.

2.6.12 Enhanced green fluorescent protein (EGFP) gene expression study

B16F10 cells were seeded 24 h prior to transfection 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/L, 10% FCS (v/v) and 1% penicillin/streptomycin) per well. Just prior to 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 (PBS) and harvested by trypsin treatment. Analysis was performed as described in (*83*) using a CyanADP flow cytometer (DakoCytomation, Kopenhagen, Denmark). 1 × 10^4 gated events were collected per sample; values are given as the average of three transfected wells.

2.6.13 *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.). At 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).

2.6.14 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;

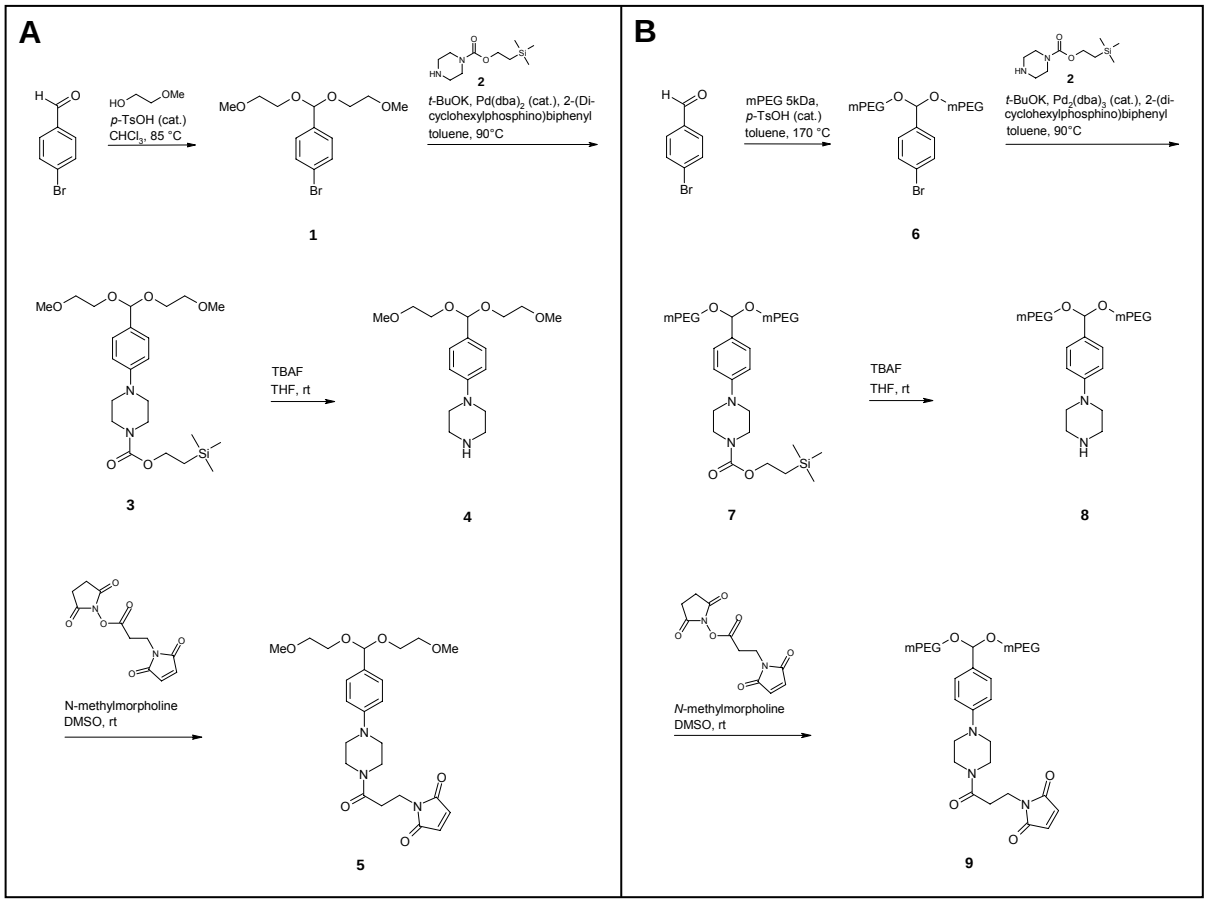
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3 Results

3.1 pH-sensitive PEGylation of polycations

3.1.1 Synthesis of PEG-A-MAL, a novel pH-sensitive PEGylation reagent

As shown in **Scheme 1A**, the initial synthesis of the acetal linker was developed with 2-methoxyethanol instead of mPEG (see supporting information). This was necessary for optimization of the synthesis as well as for the characterization of the linker by NMR, as the PEG signal overlaps several signals of the synthesis intermediates. The synthesis of the methoxyethanol acetal ME-A-MAL (**5**) was achieved in four reaction steps, each of which was confirmed by NMR analysis.



Scheme 1. (A) *Synthesis of methoxyethanol acetal ME-A-MAL (**5**) and* **(B)** *of the pH-sensitive PEGylation reagent PEG-A-MAL (**9**).*

In a similar manner the PEG acetal linker was synthesized as shown in **Scheme 1B**. In the first step *p*-bromobenzaldehyde was reacted with mPEG to generate the corresponding acetal (**6**) with a 97 % yield. Formation of the acetal was confirmed by ^{1}H NMR, by appearance of a singlet at 5.56 ppm (acetal proton) and the disappearance of a singlet at 9.96 ppm (aldehyde proton). The bromo group of **6** was then substituted with trimethylsilylethyloxocarbonyl piperazine by the Buchwald Hartwig reaction with 2-trimethylsilylethyl piperazine-1-carboxylate. ^{1}H NMR shows that the product obtained was a mixture of the piperazine substituted acetal **7** (82 %) and the starting material **6** (18 %). The signals proving the existence of **7** appear as a singlet at 0.04 ppm belonging to the nine protons of the trimethylsilyl group, a multiplet of the methylene function next to the trimethylsilyl group at 0.98-1.03 ppm and a triplet from four piperazine protons at 3.13 ppm while the signal of the other four piperazine protons is overlapped by the PEG signal. For **6** the acetal proton is represented at 5.56 ppm and the aromatic protons at 7.34-7.40, 7.45-7.52ppm. The respective signals for **7** are shifted to 5.50 ppm and to 6.87-6.92 and 7.31-7.36 ppm. The trimethylsilylethyloxocarbonyl protecting group of **7** was removed by reaction with TBAF·3H_2O, resulting in a 90 % yield. Subsequent ^{1}H NMR analysis of the reaction product **8** showed the de-protection of the amine moiety by the appearance of a signal at 2.94-2.99 ppm which corresponds to the four protons at the carbons next to the secondary amine. A 3-oxopropyl maleimido function was introduced at the deprotected amine by reacting **8** with *N*-succinimidyl-3-maleimidopropionate, resulting in a shift of the ^{1}H NMR signal from 2.94-2.99 ppm to lower field, which could not be observed due to the broad overlapping PEG signal. Furthermore a singlet appeared at 6.69 ppm, indicating the two methine protons of the maleimido group. In addition a multiplet emerged at 2.63-2.70 ppm, corresponding to the two protons next to the carboxy group of the new substituent. Product **9**, the PEGylation reagent PEG-A-MAL used in the subsequent studies, was obtained with a 71 % yield.

To ascertain the pH sensitivity of the linker, hydrolysis of **9** was carried out at pH 5.0, 5.5, 6.0, 6.5, 7.0 and pH 7.4. The assay was performed at 37 °C and the hydrolysis was determined at different time points by measuring the absorbance at 340 nm, the main absorbance of the released aldehyde. Half-life continuously decreased with decreasing pH. At pH 7.4 the acetal hydrolyzed with a half-life of approximately 1 h, while at pH 5 the half-life was less than 3 min. At a pH of 6, which is relevant for PEI

containing endosomes (*84, 85*), PEG-A-MAL degraded with a half-life of less than 10 min (**Figure 4A**). The curves in **Figure 4B** show the hydrolysis rates of PEG-A-MAL at defined time points as a function of the pH. For pH 5 at all three time points – 5, 10 and 15 min – the reagent showed almost complete degradation, while for pH 7.0 between 96 and 80 % remained intact during the indicated time periods.

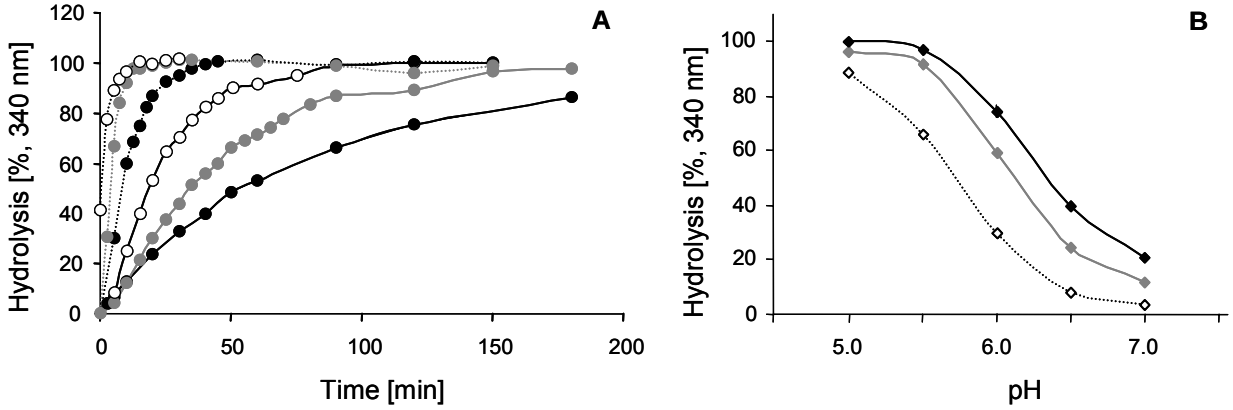
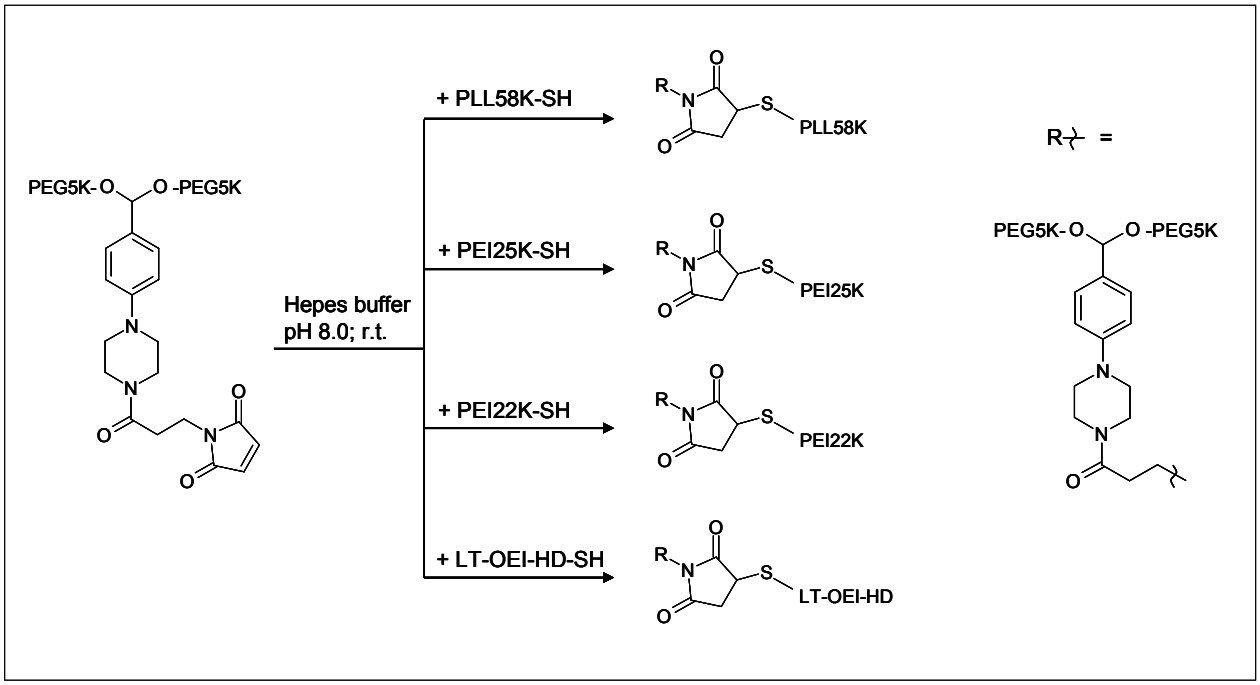


Figure 4. ***Time-course of the cleavage of PEG-A-MAL (9)*** *and formation of the corresponding aldehyde at 37 °C at different pH values (pH 7.4 , pH 7.0 , pH 6.5 , pH 6.0 , pH 5.5 , pH 5.0)* **(A)**.
Hydrolysis rate of PEG-A-MAL (9) *as a function of pH shown for different time points at 37 °C (5 min , 10 min , 15 min)* **(B)**.
Experiments were performed in duplicate. Measurement wavelength: 340 nm.

3.1.2 Reversible PEGylation of various polycations with PEG-A-MAL

Synthesis of the PEG-A-polycation conjugates was achieved by reacting the maleimide moiety of the PEG acetal linker **9** with the previously mercapto-modified polycations. Different standard polycations, such as PLL58K, branched and linear PEIs (PEI22K, PEI25K) but also the biodegradable LT-OEI-HD could be successfully reacted with the linker (**Scheme 2**). Depending on the reactivity of the different polycations used, the PEGylation degree of the resulting PEG-A-polycation conjugates slightly varied, thus about 1 to 1.5 SH groups in the thiolated polycation were required for 1 PEG-A-MAL linker in the end product. For PEG-A-PEI25K for example, about 1.5 SH groups per PEG-A-MAL were necessary. In contrast for PEI22K, the SH/PEG-A-MAL ratio was about 1/1, possibly due to a better accessibility of the thiols in the linearly structured polycation. The PEG-A-PEI25K conjugate described herein in more detail had a PEG/PEI25K molar ratio of approximately 3/1, corresponding to approximately 1.5 acetal moieties per PEI25K.



Scheme 2. ***Reversible PEGylation of variable polycations with PEG-A-MAL.*** *Thiol functionalized polycations as poly-(L-lysine) (PLL), polyethylenimine (PEI) or degradable analogs (LT-OEI-HD) were reacted with a 4-fold excess of the PEGylation reagent. Resulting PEG-A-polycation conjugates were purified on a Macro-prep High S cation exchange column.*

The hydrolytic profile of the PEG-A-polycation conjugates was exemplarily determined for PEG-A-PEI25K by measuring the absorbance at 340 nm in a similar manner as in case of PEG-A-MAL. **Figure 5** shows the time course of hydrolysis of the PEG-A-PEI25K conjugate at either pH 7.4 or pH 5.5 at 37 °C. The PEG-A-PEI25K had a half-life of approximately 3 min at pH 5.5, while the half-life was 2 h at pH 7.4.

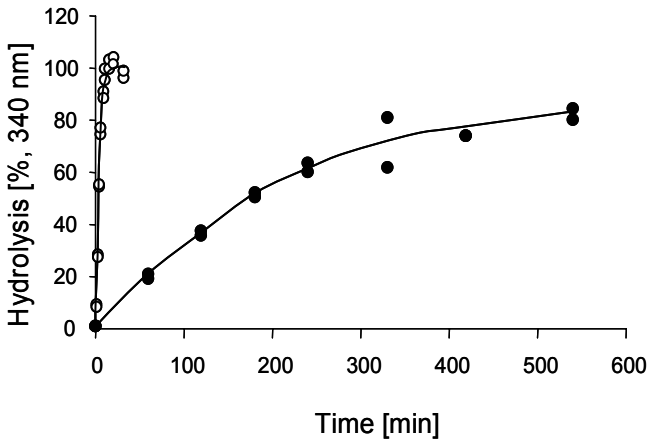


Figure 5. ***Time-course of the cleavage of PEG-A-PEI25K*** *and formation of the corresponding aromatic aldehyde at pH 7.4 (black circles) and pH 5.5 (open circles) at 37 °C. Experiments were performed in duplicates*.

To investigate the effect of PEG conjugates on the shielding of polyplexes, particle size and zeta potential were measured. For comparison: control PLL58K or PEI22K particles at N/P 6 without PEG and targeting ligands have a surface charge of about 30 mV and – in case of PEI22K – form aggregates (*81*). In preliminary experiments with polyplexes composed of either PLL58K plus PEG-A-PLL58K, PEI25K plus PEG-A-PEI25K or PEI22K plus PEG-A-PEI22K the appropriate ratios of PEGylated/non-PEGylated polycation were determined. 20-30 % (w/w PEI) (depending on the PEGylation degree and the type of the polycation) of the polycation being PEGylated turned out to be the optimal composition to achieve complete polyplex shielding at physiologic pH and quick deshielding at pH 5.0. In the next step, targeting ligands were includes into the polyplexes. Here, PLL polyplexes were not analyzed further, as PEIs are the more interesting polymers regarding gene transfer. **Table 1** shows particle sizes and zeta potential of polyplexes containing a targeting conjugate (EGF-PEI or Tf-PEI) with and without PEG shield (PEG-S-PEI25K or PEG-A-PEI25K) at pH 7.4 and 37 °C. All PEGylated polyplexes remained shielded and stable in size for 2 h, whereas non-PEGylated polyplexes formed aggregates. **Figure 6** shows the time-dependent changes of PEG-A-PEI25K shielded polyplexes incubated at either pH 5.0 or pH 7.4 and 37 °C. At pH 5.0 the PEG-A-PEI25K shielded polyplexes formed aggregates at the 0.5 h time point, indicating PEG removal under these conditions. In contrast, at pH 7.4 polyplexes were stable in size and retained low surface charge for the whole 2 h time period. Even after 4 h at pH 7.4 – representing the incubation time of cells with polyplexes – particles remained shielded (data not shown). Different targeting conjugates (EGF in **Fig. 3A, B**; Tf in **Fig. 3C, D**) did not significantly alter the biophysical properties.

Polyplexes containing the non-reversible conjugate PEG-S-PEI25K instead of PEG-A-PERI25K did not change in size and remained shielded at both pH values (**Table 1** and data not shown).

Formulation:	0 h		2 h	
70% PEI22K with:	size [nm]	zeta [mV]	size [nm]	zeta [mV]
20% PEI25K, 10% **EGF**-PEG-PEI	267	18,8	aggregates*	14,0
20% PEI25K, 10% **Tf**-PEG-PEI	aggregates*	18,2	aggregates*	13,7
20% **PEG**-**S**-PEI25K, 10% PEI25K	196	2,1	177	2,4
20% **PEG**-**S**-PEI25K, 10% **EGF**-PEG-PEI	232	3,0	251	2,7
20% **PEG**-**S**-PEI25K, 10% **Tf**-PEG-PEI	198	2,9	179	2,8
20% **PEG**-**A**-PEI25K, 10% PEI25K	204	3,2	169	3,2
20% **PEG**-**A**-PEI25K, 10% **EGF**-PEG-PEI	224	3,2	223	3,2
20% **PEG**-**A**-PEI25K, 10% **Tf**-PEG-PEI	211	3,0	203	3,1

Table 1. ***Particle size and zeta potential*** *of various polyplex formulations of N/P 6, at the 0 and 2 h time point, pH 7.4. Each value represents the mean of at least three readings. * The term "aggregates" refers to particles over 1 µm in size.*

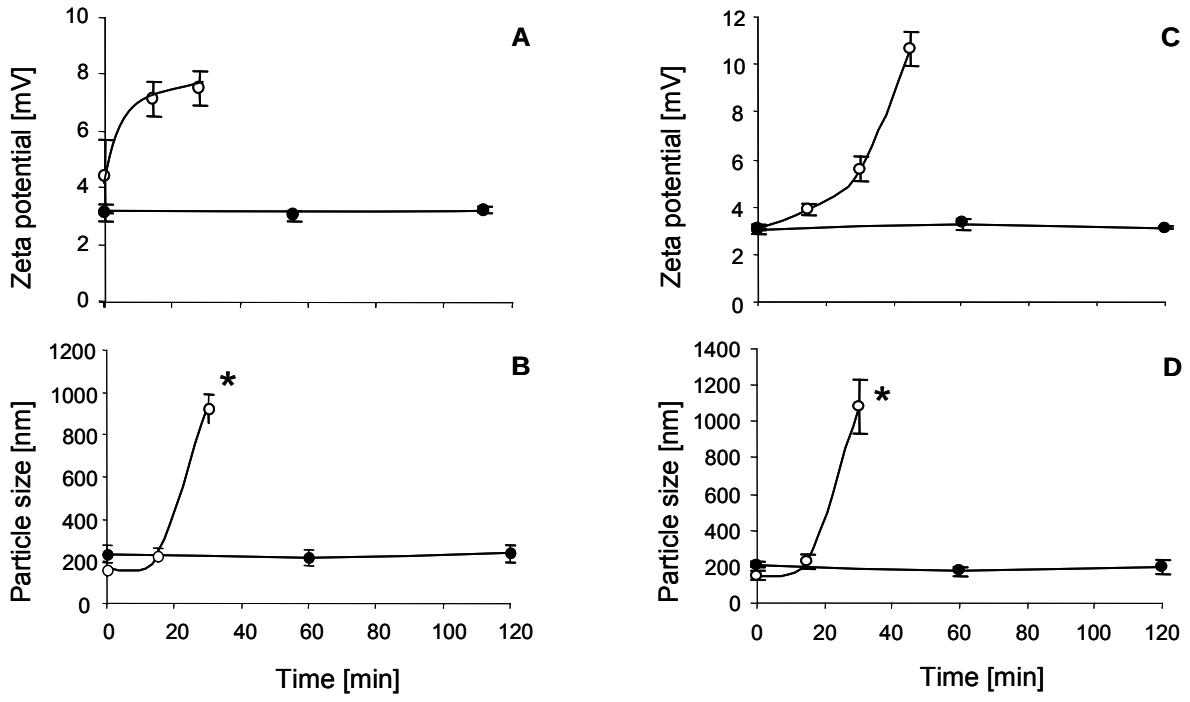


Figure 6. ***Time-course of the zeta potential (A, C) or particle size (B, D)*** *of polyplexes consisting of 70 % (w/w; PEI) PEI22K, 20 % PEG-A-PEI25K and 10 % EGF-PEI (A, B) or 10 % Tf-PEI (C, D) at pH 7.4 (closed circles) and at pH 5.0 (open circles) at 37 °C. Each point represents the mean ± s.d. of three experiments.*
** particles over 1 µm in size.*

The transfection efficiency of the various polyplex formulations was tested using a luciferase gene expressing plasmid. EGF containing polyplexes (*83, 86*) were applied on Renca-EGFR cells and Tf containing polyplexes (*74, 81*) on K562 cells. To avoid extracellular deshielding transfection medium was sucked off after 4 hours of incubation and replaced by fresh culture medium. All targeted, reversibly shielded polyplex formulations had greater gene transfer activity as compared to the analogous stably shielded formulations (see **Figure 7A** for Renca-EGFR and **Figure 7B** for K562). The effect of pH-sensitive PEGylation was most pronounced at a molar PEI nitrogen/DNA phosphate (N/P) ratio of 3 in Renca-EGFR, with a 14-fold enhanced gene expression compared to non-sensitive PEGylation (**Figure 7A**). Analog, for K562 cells the effect of pH-sensitive PEGylation was most pronounced at a molar N/P ratio of 6 resulting in 8-fold enhancement of gene expression (**Figure 7B**). As polyplexes remained shielded within the incubation time, the higher transfection efficiency of reversibly shielded compared to stably shielded polyplexes must be due to intracellular deshielding and not to an increased uptake because of extracellular deshielding.

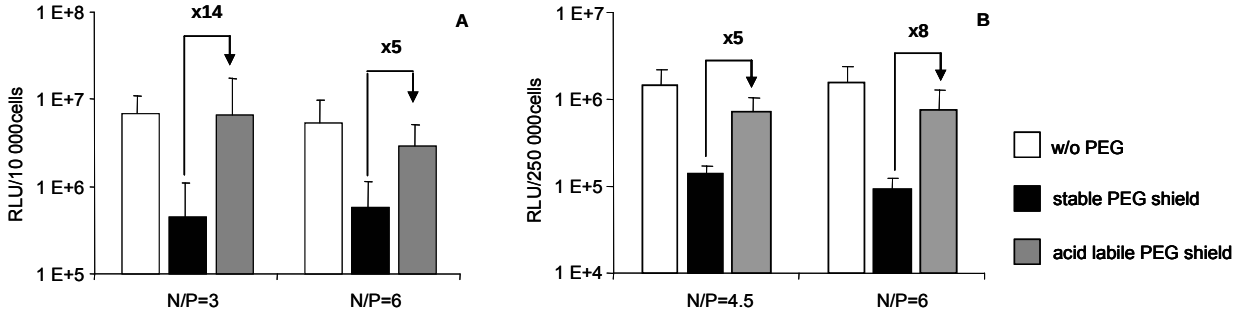


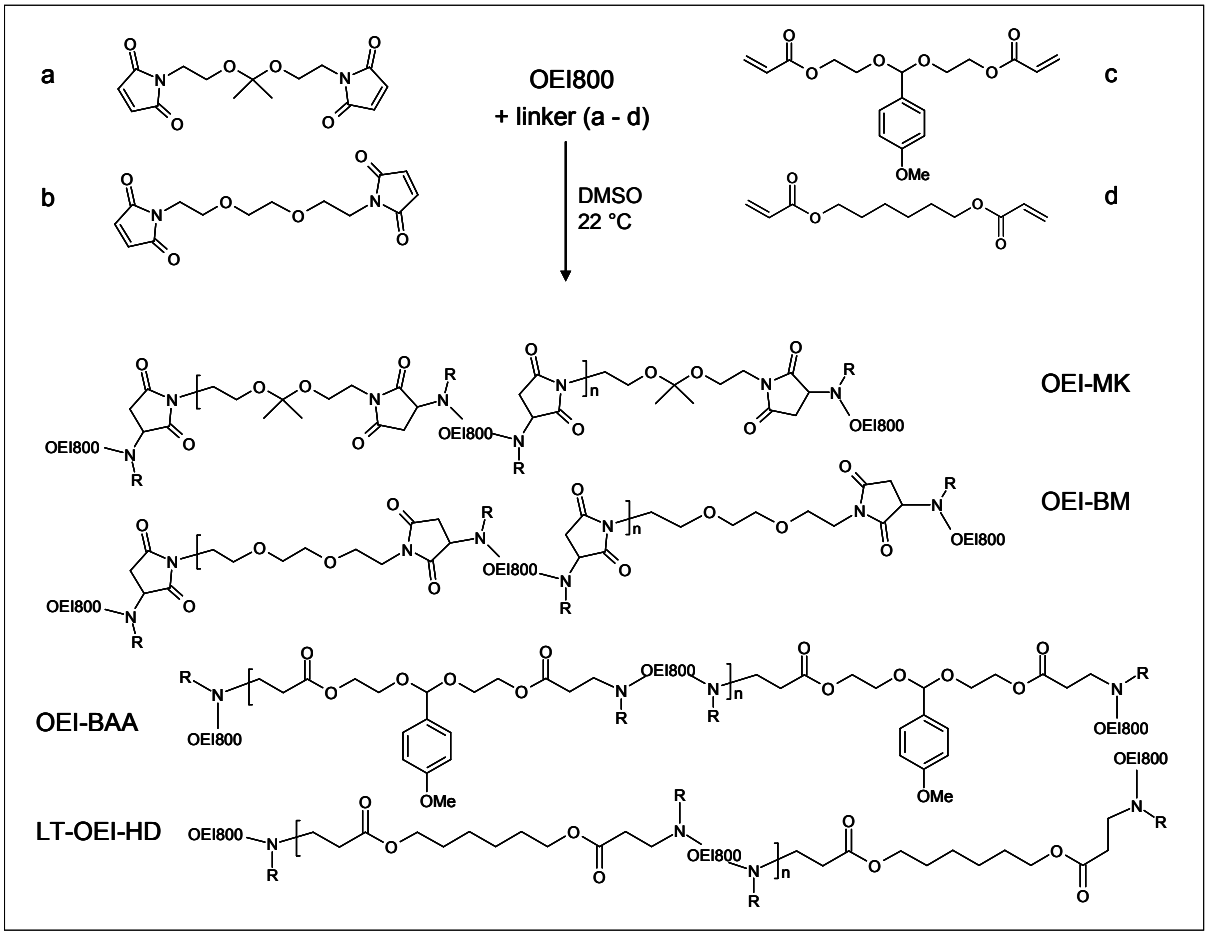
Figure 7. ***Luciferase gene expression*** *by EGF-receptor-targeted polyplexes on* ***Renca-EGFR cells (A)*** *and by transferrin-receptor-targeted polyplexes on* ***K562 cells (B)****.*
Polyplexes were composed of 70 % PEI22K, 10 % targeting ligand (EGF-PEI or Tf-PEI respectively) and 20 % of either PEI25K (nonshielded polyplexes, white bars), a stable PEG-PEI25K conjugate (stably shielded polyplexes, black bars) or PEG-A-PEI25K (reversily shielded polyplexes, gray bars). Each bar represents the mean ± sd of three individual experiments, each performed in triplicate.

3.2 pH-sensitive biodegradable cationic carriers

3.2.1 Acid-degradable bisacrylate acetal (BAA) and maleimido ketal (MK) polymers and acid-insensitive HD and BM control polymers

3.2.1.1 Polymer syntheses and structural analysis

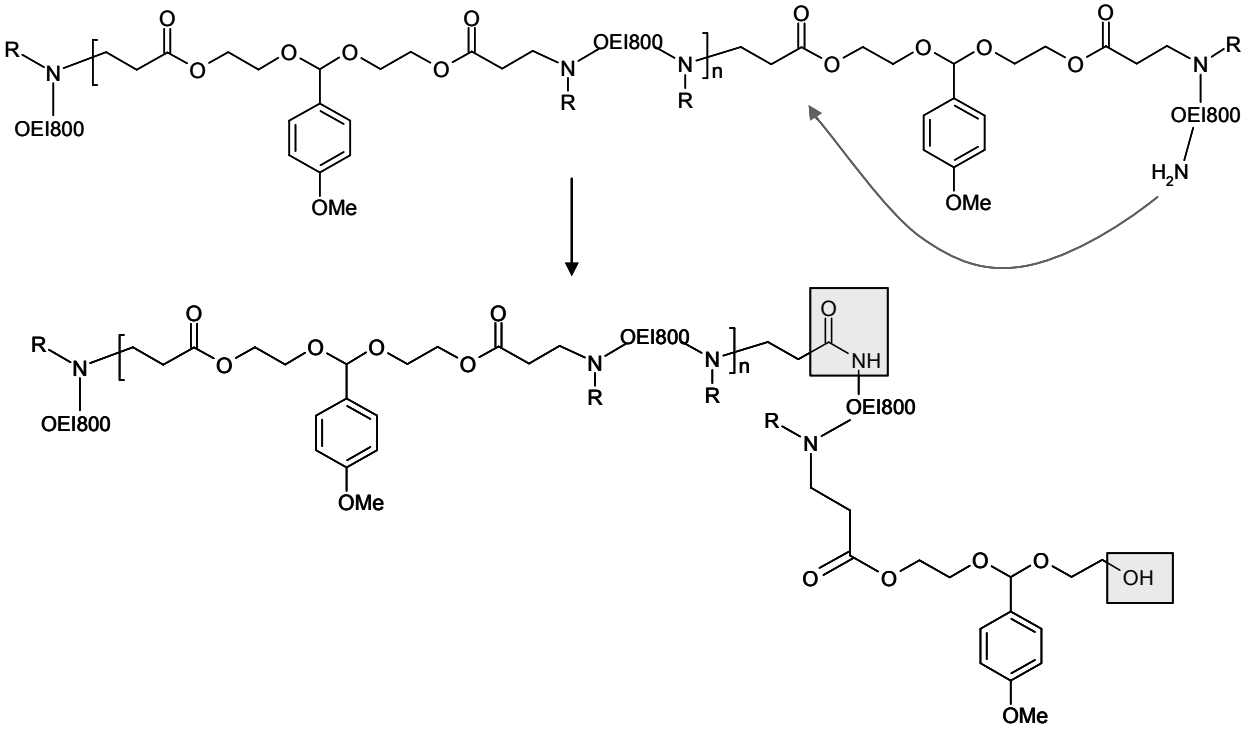
The control polymer LT-OEI-HD (1,6-hexanediol diacrylate linked OEI800 synthesized at low temperature (LT), i.e. 22 °C) was synthesized as described by Kloeckner et al. (*79*). All polymers were generated by reacting OEI800 via its amine functions with bifunctional linkers in a Michael addition (**Scheme 3**). In case of maleimido-functionalized linkers (MK and BM) reaction time was 22 hours, while for acrylate linkers different reaction times were chosen: 4 days in case of LT-OEI-HD (HD linker is not as amenable to aminolysis as BAA linker), 45 min, 1.5 days or 3 days in case of BAA polymers. The Michael addition between BAA linker and OEI800 is complete after 45 minutes, therefore an elongation of the reaction time causes only higher extents of aminolysis. Polymers synthesized from maleimido linkers (BM and MK) in contrast, do not show this side-reaction. Therefore, changes in the reaction time do not influence the chemical structure of the products here, as it is the case for the acrylate linked polymers. All syntheses were carried out in anhydrous DMSO at room temperature (22 °C) to prevent degradation of the acetal and ester functions present in the linker moieties (*79*). The nomenclature of the novel polymers (i.e. MK-A1/1, BM-A1/1, BAA-1/1-3d, -2/1-3d, …) is composed as follows: first, the applied linker (MK, BM, …) is specified, in case of application of different monomeric units followed by an abbreviation thereof (A for OEI800, B for PEI1.8K); secondly, the linker/OEI molar input ratio (1/1, …) is noted; in case of BAA polymers where reactions were carried out over different periods of time, the reaction time is mentioned at the end.



Scheme 3. ***Syntheses of the oligoethylenimine based polymers:*** *acid-degradable acetal-linked OEI-MK and OEI-BAA polymers and acid-nonsensitive control polymers OEI-BM and LT-OEI-HD.* ***a****: acid-labile MK-linker;* ***b****: acid-stable BM-linker;* ***c****: acid-labile BAA-linker;* ***d****: acid-nonsensitive HD-linker. (The more general names "OEI-MK", "OEI-BAA", etc. used here stand representatively for the whole groups of MK-, BAA or BM-linked polymers)*

Purification of the products from the solvent and from unreacted starting material was achieved by dialysis at 4 °C using a 3.5 kDa molecular mass cut off membrane. After freeze-drying BM-A1/1 and MK-A1/1 were obtained as red solid in yields of 48 % and 40 %, respectively. BAA polymers and LT-OEI-HD gave colorless solids in 40 % (BAA-1/1-3d), 50 % (BAA-2/1-3d), 56 % (BAA-1/1-1.5d), 70 % (BAA-1/1-45m), 58 % (BAA-1/1-45m-hc) and 65 % (LT-OEI-HD) yield. Polymers were then analyzed by ^{1}H NMR to investigate their chemical structure (OEI to linker ratio) and to confirm the preservation of the labile acetal functions during synthesis and purification. For BM-A1/1 a linker to OEI ratio of 1.3/1 was found. For MK-A1/1 the linker to ketal to OEI ratio was 1.03/0.93/1, indicating a ≤10 % hydrolysis of ketal groups in the product

during workup. LT-OEI-HD had a HD to OEI ratio of 1.25/1, with 77 % of esters remaining intact. The slightly reduced ester content is not triggered by hydrolysis, but can be ascribed to low-level partial exchange reaction, i.e. aminolysis by OEI amines during the synthesis in water-free DMSO, and does not negatively affect the polymer size (*79*). For BAA polymers which are more sensitive towards aminolysis (**Scheme 4**), the content of intact diester in the linker molecules was 26 % for BAA-1/1-3d and BAA-2/1-3d; if the reaction was finished already after 1.5 days (BAA-1/1-1.5d) a diester content of 40 % was maintained. Reduction of the reaction time to only 45 min increased the diester content in the linker to 75 % (BAA-1/1-45min) and 60 % (BAA-1.25/1-45min-hc).



Scheme 4. ***Aminolysis of BAA polymers:*** *amine functions of OEI800 moieties react intra- or intermolecularly with the ester fuctions of the polymer, resulting in stable amide bonds.*

For the better understanding of the amide formation during the synthesis process (**Scheme 4**), the synthesis of BAA-1/1 has been additionally performed in DMSO-d_6 as solvent, so that the reaction could be followed by ^{1}H NMR. By analyzing the signal of the acetal protons (5.6 - 5.5 ppm) the ratios of dieester/monoester/diol could be investigated (**Figure 8A**). The process of aminolysis of the ester bonds over

time, and therefore the dependence of diester content from the reaction time is illustrated in **Figure 8B**.

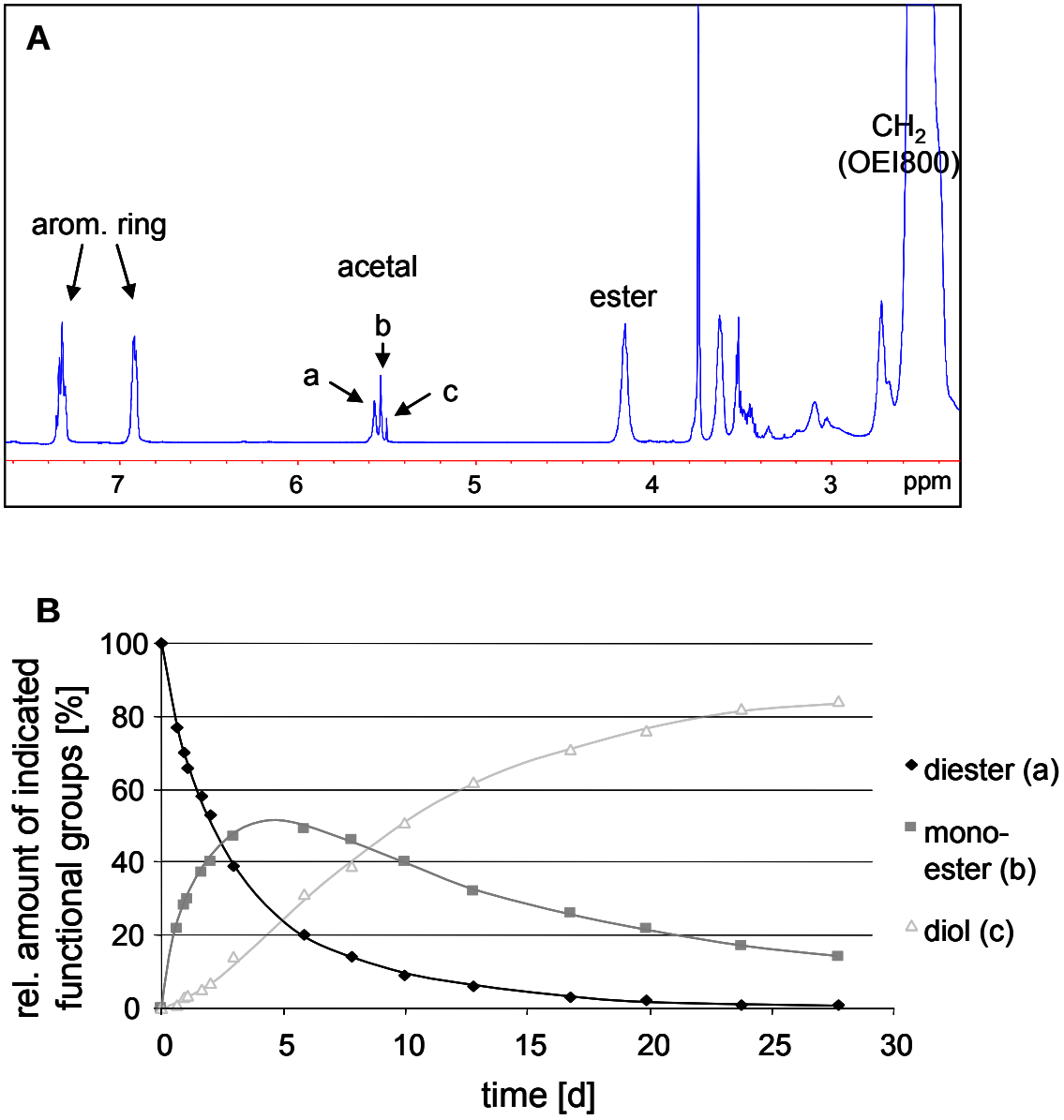
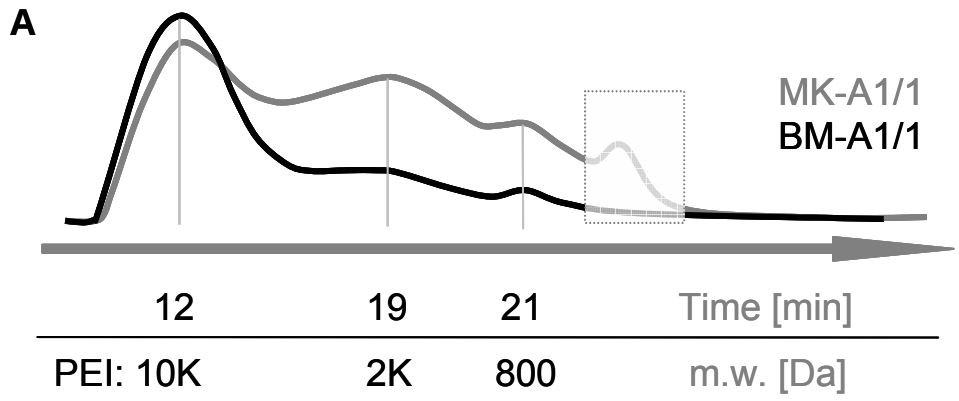


Figure 8. ***Structural analysis of BAA-1/1 during polymer synthesis at 22 °C in DMSO-d_6:*** *^{1}H NMR spectra (**A**) were recorded at different time points (shown here: reaction time 48 h) during the reaction and the degree of aminolysis was calculated on the basis of the acetal signal (5.6 - 5.5 ppm). This signal shows three different chemical shifts: for the intact diester it is located at 5.57 ppm (a), for the monoester (aminolysis on one side of the linker) it is shifted slightly to the higher field and appears at 5.54 ppm (b), while in case of the diol (aminolysis on both linker sides) it appears as a peak at 5.50 ppm (c).*
*Diagram of the ^{1}H NMR based kinetics of BAA aminolysis over 28 days (**B**).*

Infrared analysis of BAA-1/1-45min, BAA-1/1-1.5d and BAA-2/1-3d additionally confirmed the presence of amides (amide bond: 1645 cm^{-1}) in the polymers as well as the increase of amide and decrease of ester bonds (ester bond: 1732 cm^{-1}) with the elongation of reaction time.

An estimate of molecular weight (m.w.) of the polymers was obtained by size exclusion chromatography (**Figure 9**) using Superdex 75 for the separation in the low m.w. range and Superdex 200 one for separation in the high m.w. range. As running buffer 20 mM Hepes containing 0.25 M NaCl, adjusted at pH 7.4 was used. At this pH, acid-labile functions as well as esters are sufficiently stable during the run. Calibration with standard polymers of known m.w. and of comparable chemical structure, OEI800, PEI2K, PEI10K, PEI25K and PEI50K, was performed. **Figure 9** shows the elution profiles of polymers compared with the elution times of the standards. For all polymers a broad size distribution was found. In case of BM-A1/1 and MK-A1/1 the main peak maximum in the elution profiles was located at about 12 minutes on Superdex 75 (**Figure 9A**) and 20 minutes on Superdex 200 (**Figure 9B**), indicating a m.w. of about 10 kDa or slightly less. While this main maximum was the same for the both polymers, the average size of BM-A1/1 was slightly higher than that of MK-A1/1: in MK-A1/1 low m.w. fractions are present to a higher extent than in BM-A1/1.



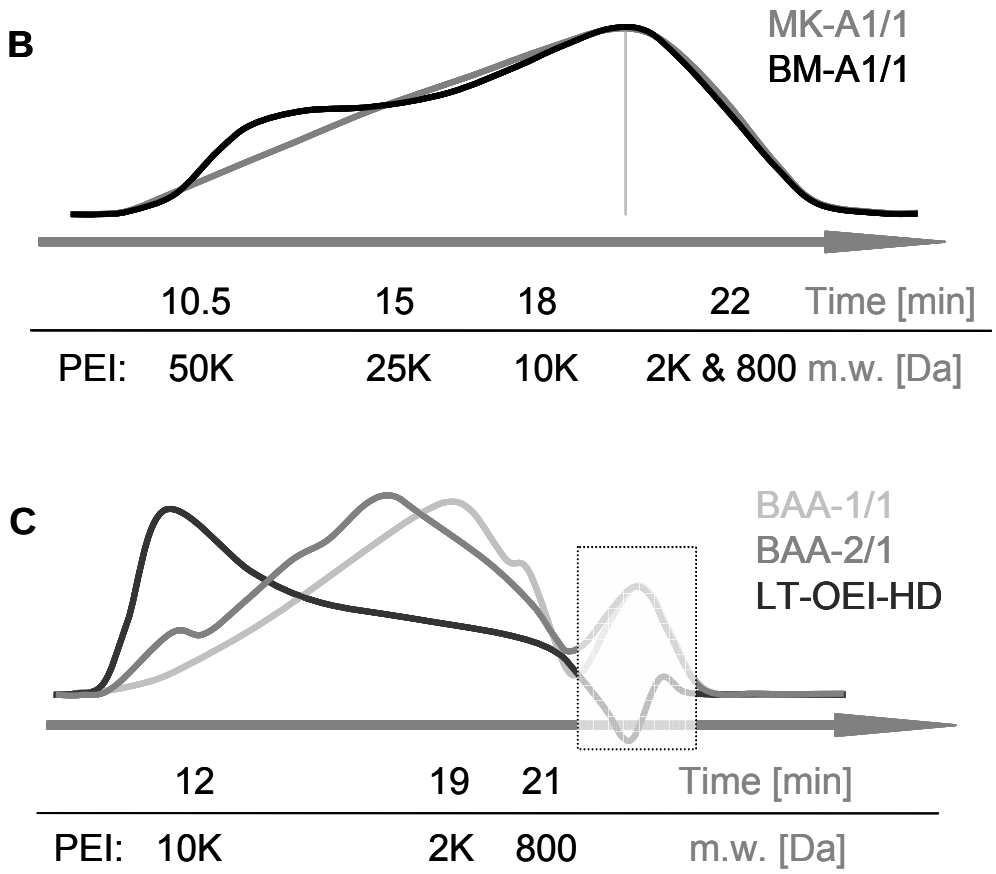
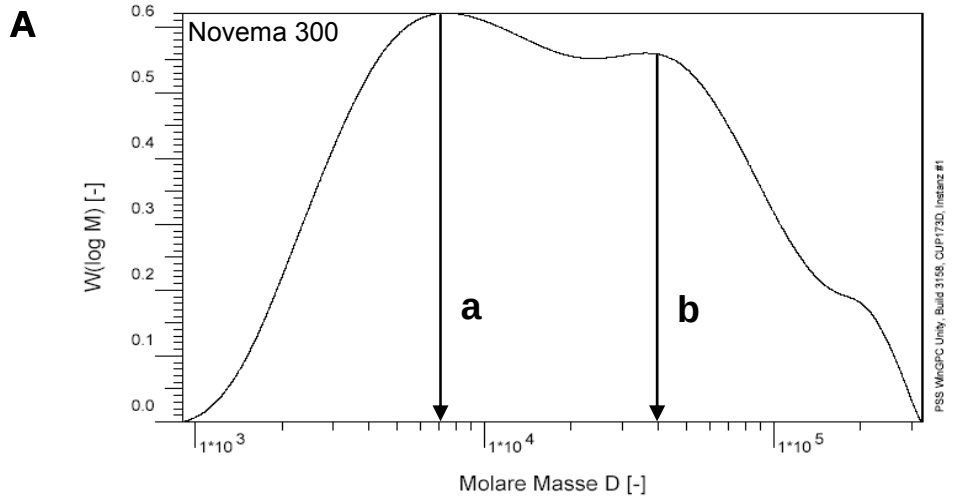


Figure 9. ***Investigation of polymer molecular weight by size exclusion chromatography (SEC).*** *Elution profiles of MK-A1/1 and BM-A1/1 on a Superdex 75 column (**A**) for fractionation in the low m.w. range, and on Superdex 200 for separation in the high m. w. range (**B**). Elution profiles of BAA polymers and LT-OEI-HD on Superdex 75 (**C**). Peaks at the very right side of the diagrams (marked by shaded boxes) were induced by salt and do not refer to the samples.*

When analyzed on Superdex 200, which separates in the higher m.w. range, another smaller maximum can be found for BM-A1/1 at about 12 minutes – thus lying slightly below the 50 kDa standard (10.5 min) (**Figure 9B and 10A lower panel**). Additional analysis of BM-A1/1 by gel permeation chromatography on a Novema 300 column (**Figure 10A, upper panel**) showed a maximum in the lower m.w. range at 7 kDa (a) and a second one in the higher m.w. range at 40 kDa (b). These results are well comparable to those obtained from Superdex 200. The average values calculated for BM-A1/1 from the Novema analysis resulted in Mn = 8.5 kDa and Mw = 36 kDa with a polydispersity of 4.2.

A


Novema 300
W(log M) [-]
0.6
0.5
0.4
0.3
0.2
0.1
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a
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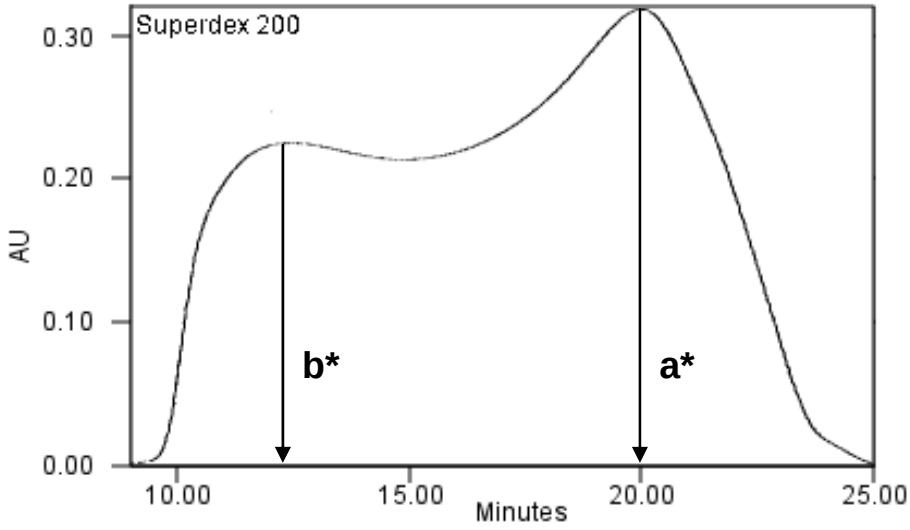
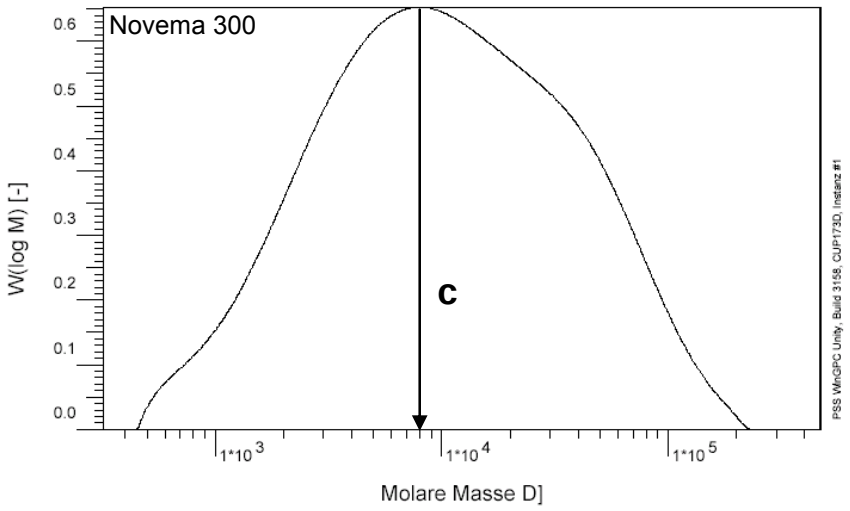
1*10 3
1*10 4
1*10 5
Molare Masse D [-]
PSS WinGPC Unity, Build 3158, CUP173D, Instanz #1


Superdex 200
AU
0.30
0.20
0.10
0.00
b*
a*
10.00
15.00
20.00
25.00
Minutes

B


Novema 300
W(log M) [-]
0.6
0.5
0.4
0.3
0.2
0.1
0.0
c
1*10 3
1*10 4
1*10 5
Molare Masse D]
PSS WinGPC Unity, Build 3158, CUP173D, Instanz #1

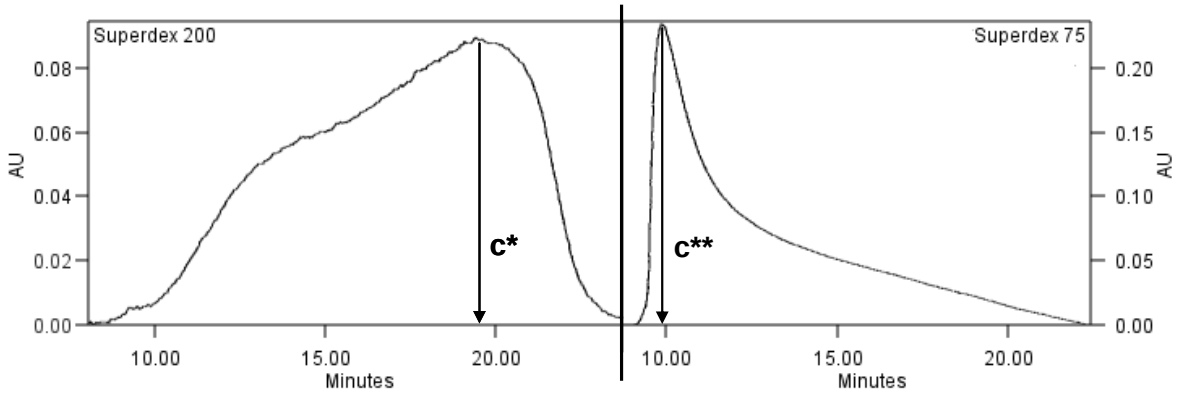


Figure 10. ***Investigation of polymer molecular weight by GPC and SEC. Comparison of results obtained on a Novema 300 column with those received on Superdex columns.***
(**A**) *BM-A1/1:upper panel, Novema 300 (a: 7 kDa; b: 40 kDa); lower panel, Superdex 200 (a*: 20 min, corresponding to 8-10 kDa; b*: 12 min, corresponding to about 40 kDa)*
(**B**) *LT-OEI-HD: upper panel, Novema 300 (c : 8 kDa); lower panel, Superdex 200 (c*: 19 min, corresponding to 8-9 kDa); Superdex 75 (c**: 10 min, corresponding to 10 kDa)*

Polymer	**SEC**			**GPC**				
	Mp	m.w. range	Vp	Mp	Vp	Mn	Mw	PDI
BM-A1/1	8-10 kDa, 40 kDa	800 Da - >50 kDa	12 mL (Sup. 200), 20 mL (Sup. 200)	7 kDa 40 kDa	10.3 mL	8.5 kDa	36 kDa	4.2
MK-A1/1	8-10 kDa	800 Da - 50 kDa	20 mL (Sup. 200)					
LT-OEI-HD	8-10 kDa	800 Da - 50 kDa	20 mL (Sup. 200)	8 kDa	10.1 mL	4.9 kDa	21 kDa	4.3
BAA-2/1-3d	4 kDa, 8-10 kDa	800 Da - 10 kDa	20 mL (Sup. 200); 12, 14, 17mL (Sup. 75);					
BAA-1/1-3d	2-3 kDa	800 Da - 10 kDa	18.5 mL (Sup. 75)					
BAA-1/1-1.5d	2-3 kDa	800 Da - 10 kDa	18.5 mL (Sup. 75)					
BAA-1/1-45min	2-3 kDa	800 Da - 8 kDa	18.5 mL (Sup. 75)					
BAA-1.25/1-45min-hc	2-3 kDa	800 Da - 9 kDa	18.5 mL (Sup. 75)					

Table 2. ***Overview over the key data obtained by SEC and GPC.*** *Polymer size characterization by SEC, and in case of acid-stable polymers also by GPC: m.w. at peak maximum (Mp), m.w. ranges, elution volume at peak maximum (Vp), molecular weight by number (Mn) and by weight (Mw), polydispersity index (PDI). For SEC experiments Vp was calculated from the elution times in consideration of the flow rate (1 mL/min).*

A broad polydispersity was also found for BAA polymers and LT-OEI-HD. Analysis on Superdex columns showed that these polymers were smaller in size compared to MK-A1/1 and BM-A1/1. Among the acrylate linked polycations LT-OEI-HD was the

largest with a peak maximum slightly below the 10 kDa standard (**Figure 10B,** lower panel). As LT-OEI-HD is acid-stable it was additionally analyzed on a Novema 300 column (**Figure 10B,** upper panel). Here the peak maximum appeared at a molar mass of about 8 kDa (c), which is in good accordance with the Superdex 200 and 75 results, where the maxima in the elution profiles were located at 19 (c*) or 10 minutes (c**) respectively, indicating m.w. of 10 or slightly below 10 kDa (for elution profiles of standards see also **Figure 9B and C**). For LT-OEI-HD the values calculated from Novema analysis were Mn = 4.9 kDa and Mw = 21 kDa with a polydispersity of 4.3.

BAA polymers showed SEC peak maxima in the range of 2-3 kDa and size distributions reaching from the monomer up to 8-10 kDa. Thereby, a differentiation between polymers with higher and lower linker content was possible: compared to the BAA-1/1 polymers, the BAA-2/1-3d elution profile is slightly shifted to the higher m.w. fractions (**Figure 9C**).

Based on the good correlation of SEC and GPC data and the fact that the established GPC method required the use of pH 2.8 mobile phase, m.w. distribution of the pH-labile polymers were analyzed by SEC only. A detailed overview over the data is given in **Table 2**.

3.2.1.2 Chemical and biophysical properties

Degradation study of MK-A1/1 and BAA polymers. To evaluate the pH dependency of the hydrolysis of the acid-labile linkers in MK-A1/1 and BAA polymers, degradation kinetics were measured in incubations at body temperature (37 °C) at physiological pH 7.4 as well as at pH 5.0 (**Figure 11**). The half-life of MK-A1/1 was about 5 h at pH 7.4 (**Figure 11A**), while at pH 5.0 the polymer hydrolysis was almost complete (98 %) after 15 min. In contrast, control polymer BM-A1/1 was stable even after 20 h of incubation at pH 5.0 and 37 °C (data not shown). Degradation of the acetal function in BAA polymers was analyzed in more detail by measuring the increase of UV-absorbance of the released aromatic benzaldehyde. Here, at pH 5.0 hydrolysis proceeded with a half-life of about 3 minutes and was complete in less than 30 min (**Figure 11B**) while at pH 7.4 the half-life was about 3 ½ hours (**Figure 11C**). The slower hydrolysis of the ester bonds (see below) is not detectable in this assay.

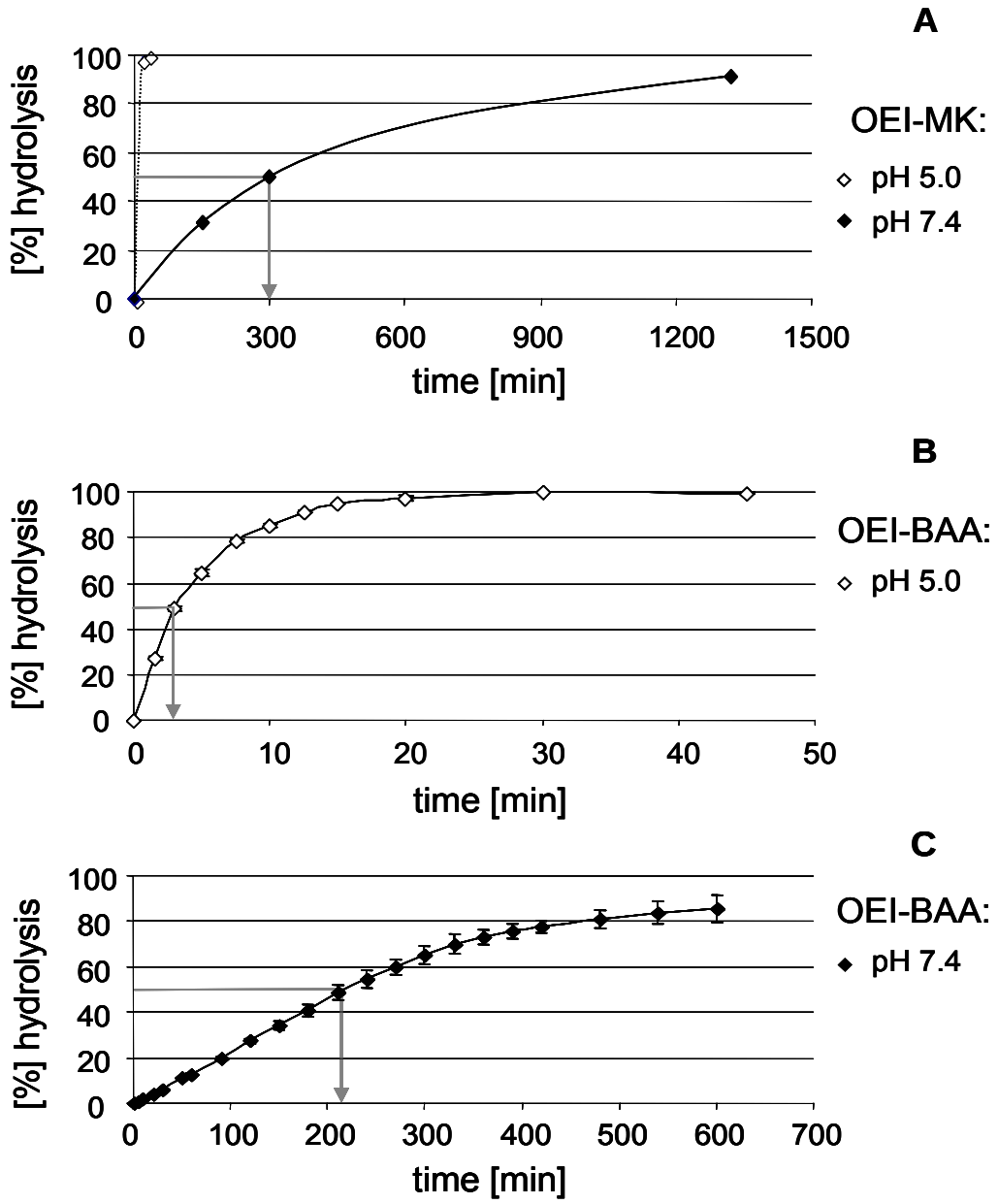


Figure 11. ***Hydrolysis kinetics of acid-labile polymers at 37 °C.*** *Grey arrows mark the half-lives of the polymers. Degradation of MK-A1/1 at physiological pH, values calculated from HPLC data (Superdex 75; AUC at different time points after incubation) (**A**). Hydrolysis kinetics of BAA polymers at pH 5.0 (**B**) and pH 7.4 (**C**), 37 °C. UV absorbance (300 nm) of the arising hydrolysis product was measured at indicated time points. Each point represents the mean ± sd of three experiments.*

Dependent on their different acetal and amide contents BAA polymers undergo acid catalized hydrolysis to varying extents, which is reflected in hydrolysis end-products of different sizes. In a hydrolysis study by SEC (Superdex 75), the degree of this pH dependent degradation was investigated. BAA-1/1-45min, the polymer with the highest diester – and thus acetal linker – content, degraded completely into

monomers besides a fraction of remaining dimers. BAA-1/1-1.5d which possesses less acetal linkers showed similar results, but here the degradation products were shifted more to the dimers compared to the 45min-polymer. Additionally a marginal amount of oligomers remained. BAA-2/1-3d, in contrast showed considerable amounts of remaining oligomers after acid treatment. Even despite the higher amide content of this polymer hydrolysis still took place, especially in the high m.w. range of the polymer, indicated by the flattening of the elution profile in this area.
Degradation of ester bonds was analyzed by ^{1}H-NMR using the ester-bond containing control polymer LT-OEI-HD at pH 7.4, 37 °C. The half-life was found to be 2 days. Under acidic conditions the ester-based polymers would hydrolyze significantly slower, as reported by Kloeckner et al. (*79*).

DNA-binding assay. All polymers were analyzed for their DNA binding affinity by ethidium bromide (EtBr) exclusion assay in HBG (**Figure 12**). The assay revealed that the presence of MK or BM linker does not hamper DNA binding. In contrast these polymers bound to DNA slightly stronger than native OEI800. For BAA polymers the binding capacity was slightly reduced compared to OEI800; for LT-OEI-HD this was the case only at low c/p ratios, while with increasing c/p DNA binding became stronger than for OEI800.

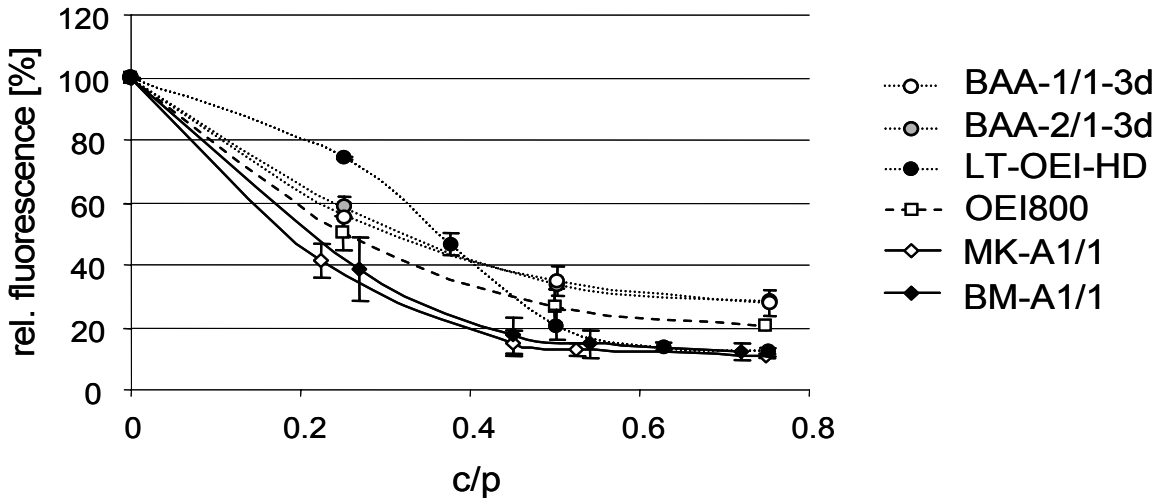


Figure 12. ***DNA-binding affinity of OEI800 and OEI800 based polymers*** *determined by ethidium bromide exclusion assay in HBG pH 7.4. The diagram shows the influence of the different linkers on DNA condensing ability of the polymers. For BAA polymers only BAA-1/1-3d and BAA-2/1-3d are shown. The curves of the three other BAA polymers were according. Each point represents the mean ± sd of three experiments.*

Particle size and zeta potential. To further characterize the polymers with regard to their use as gene vectors, complexes with pDNA were formed at different c/p ratios. These polyplexes were analyzed for their size and surface charge. Additionally, the influence of ionic strength on particle stability was investigated. In HBG polymers formed polyplexes of small size (BM-A1/1, MK-A1/1: 130 - 200 nm; LT-OEI-HD, BAA polymers: 150 – 300 nm) and positive surface charge (23 - 30 mV) at c/p ratios of 1 and more (**Figure 13**), with the exception of BAA-1/1-3d where polyplexes aggregated with time. For c/p ratios of 0.5 or 0.25 surface charges of polyplexes became lower or even negative and also for the other polymers particles tended to aggregate. Upon the addition of salt (HBS), aggregation of particles occurred in case of polymers with weaker DNA binding ability, i.e. BAA polymers (all c/p ratios tested) and LT-OEI-HD (for c/p ratios of 1 or less).

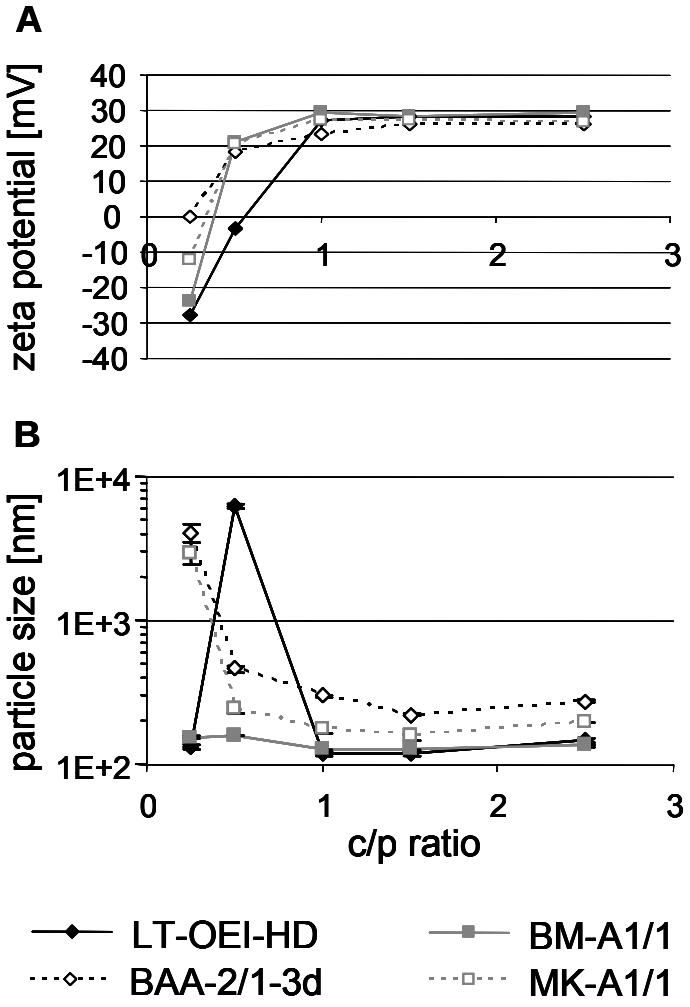


Figure 13.

Size and surface charge *of polyplexes made from MK-A1/1, BM-A1/1, BAA-2/1-3d and LT-OEI-HD at different c/p ratios (in HBG, pH 7.4). All polymers formed small particles with positive surface charge from c/p 0.5 or 1.0 (LT-OEI-HD) on. Experiments were performed in triplicate, each point represents the mean ± sd.*

3.2.1.3 In vitro luciferase reporter gene expression and metabolic activity of transfected cells

In order to investigate the influence of polymer acid degradability on transfection efficiency and cytotoxicity, B16F10 (**Figure 14**) and Neuro2A cells (**Figure 15**) were transfected with pCMVLuc reporter gene polyplexes prepared in HBG with the acid-labile polycations or their acid-insensitive counterparts at different c/p ratios. Linear PEI of 22 kDa (PEI22K) previously established as our golden standard was used for comparison at its optimal c/p ratio (c/p 0.78, corresponding to N/P 6) (*64*).

For B16F10 cells, the maximum transfection efficiency of both degradable MK-A1/1 and its stable analog BM-A1/1 reached values of about 1 log unit below the golden standard PEI22K applied at c/p 0.78 (**Figure 14A**). For maximum efficiency, higher c/p ratios (5 and more) of MK-A1/1 were required than for BM-A1/1 (optimal transfection at a c/p ratio of 2.5). For BM-A1/1 further increase in c/p resulted in a reduction of transfection efficiency. This can be explained by significant cytotoxicity (**Figure 14B**) caused by BM-A1/1 at c/p 5 and higher. In contrast, no cytotoxicity was observed for pH-labile MK-A1/1 up to c/p 7.5. Consequently, at high c/p ratios the acid-labile polymer MK-A1/1 became superior. The starting material OEI800 shows no cytotoxicity but also no transfection efficiency, independent from the c/p ratio. The golden standard PEI22K shows only moderate cytotoxicity at optimal c/p ratio of 0.78, but very strong toxicity at higher c/p ratios. Thus, relative metabolic viability of B16F10 cells transfected with PEI22K decreased to less than 50 % for c/p 2 and to less than 40 % for c/p 4 as shown by Russ et al., (*64*) in their supplementary information.

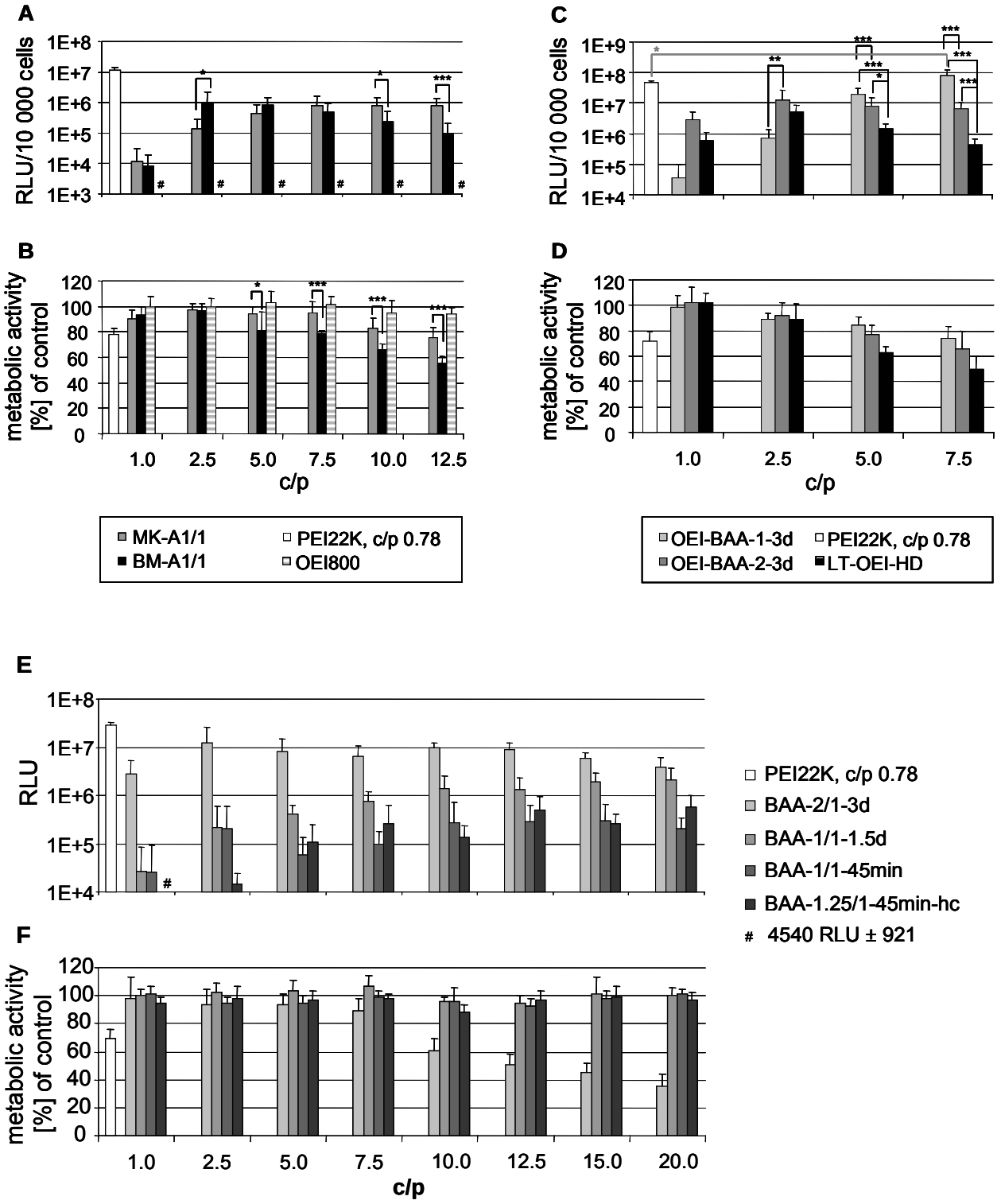


Figure 14. In vitro *transfection efficiency* *(luciferase assay, **A, C, E**) and **metabolic activity** (MTT assay, **B, D, F**) in B16F10 cells. Cells were treated with polyplexes of MK-A1/1 and BM-A1/1 in comparison to OEI800 and PEI22K (**A**, **B**), or with BAA and LT-OEI-HD (**C**, **D**). Diagrams **E** and **F** show the influence of acetal linker content on transfection efficiency and toxicity of BAA polymers.*
Each bar represents the mean ± sd of three individual experiments, each performed in triplicate. # in figure A: OEI800 < 10^3 RLU for all c/p ratios tested.

The BAA polymers and its acetal-free ester analog LT-OEI-HD (**Figure 14C, D**) were also evaluated on B16F10 cells. Here, BAA-1/1-3d was the most promising polymer, with an optimal transfection efficiency (at c/p of 7.5) significantly exceeding even PEI22K (**Figure 14C**). The acetal-free control polymer LT-OEI-HD could not reach the transfection levels of BAA-1/1-3d and BAA-2/1-3d. Although the ester-based degradable polymer LT-OEI-HD is far less cytotoxic than PEI22K, it showed considerable toxicity at c/p $\geq$ 5. The acetal- and ester-containing BAA counterparts showed the best biocompatibility among the four polymers tested (**Figure 14D**). Again it was especially at the higher c/p ratios (5.0, 7.5), where the benefit of the BAA polymers was clearly visible, not only in terms of improved compatibility but also regarding superior gene transfer efficiency.

To investigate the influence of diester-, and therefore also acetal-content in BAA polymers on the transfection efficiency/cytotoxity profiles, several BAA polymers that differ in their linker content were compared. BAA-2/1-3d, BAA-1/1-1.5d, BAA-1/1-45min and BAA-1/1-45min-hc were chosen, as they provide polyplexes of comparable size and surface charge. To see significant toxic effects, it was necessary to increase the c/p ratios in this experiment. A clear relationship between the amount of acid degradable linker in the polymer and its cytotoxicity could be found: although exhibiting better biocompatibility than acetal-free LT-OEI-HD (**Figure 14D**), BAA-2/1-3d – the polymer with the poorest degradability among the BAAs – became toxic at higher c/p ratios. This was not the case for polymers with higher acetal content (BAA-1/1-1.5d and the two 45min variants), where no toxicity occurred at all, even at the highest c/p ratio tested (**Figure 14F**). Concerning transfection efficiency (**Figure 14E**), the polymers with the lowest toxicity showed also the lowest transfection levels. While clearly superior to OEI800, BAA-1/1-45min and BAA-1/1-45min-hc could not reach the efficiency of BAA-2/1-3d or BAA-1/1-1.5d. Thereby, BAA-1/1-1.5 was inferior to BAA-2/1-3d at low c/p ratios but became almost equal at higher concentrations, while still being completely non-toxic.

Transfections were also carried out with Neuro2A cells as a second cell line. In this cell line gene transfer efficiencies of all polyplexes (including also the standard PEI22K) prepared in HBG buffer were low (**Figure 15A**, solid bars). In contrast, transfection efficiency was improved for several polymers if formulations were generated in salt-containing HBS buffer (**Figure 15A**, striped bars). All BAA polymers (including also those not shown in figure 7) and PEI22K responded to this

procedure with strongly (approximately 100-fold) elevated transfection levels. Cytotoxicity (**Figure 15B**) was not influenced remarkably by the presence of salt, except for PEI22K where metabolic activity of treated cells decreased by 20 %. Interestingly, HBS had only little influence on the transfection efficiency of LT-OEI-HD and no influence on MK-A1/1 and BM-A1/1 polyplexes (data not shown).

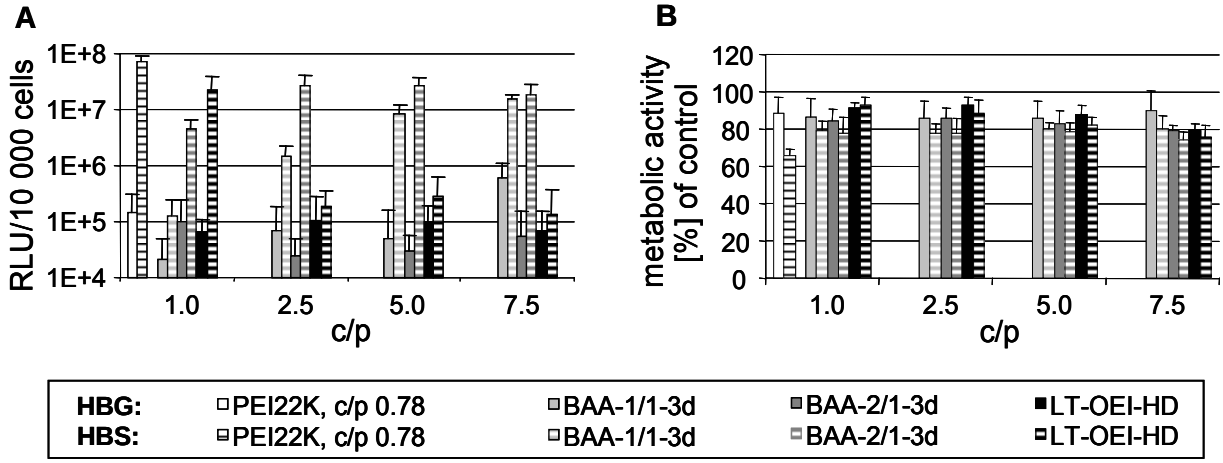


Figure 15. ***Influence of ionic strength*** *(solid bars: salt free HBG; striped bars: 150 mM NaCl containing HBS)* ***on the*** **in vitro** ***transfection efficiency*** *(luciferase assay,* ***A****)* ***and toxicity*** *(MTT assay,* ***B****) in Neuro2A cells, a cell line sensitive towards particle size. Just like PEI22K, also BAA polyplexes are influenced by the addition of salt, which leads to particle growth and therefore increased transfection efficiency. Each bar represents the mean ± sd of three individual experiments, each performed in triplicate.*

To get some insight into intracellular behaviour and transfection characteristics of BAA and MK polymers, transfection experiments were additionally performed with representative polymers using bafilomycin A1, a proton pump inhibitor. It is known that, by inhibiting the endosomal acidification, this reagent decreases transfection efficiency of polymers with high buffering capacity like PEI (*49*). In contrast, it has no significant influence on transfection efficiencies of polymers lacking this property (for example PLL) (*87*). For this study B16F10 cells were transfected either in presence or absence of bafilomycin A1 with MK-A1/1, BAA-2/1-3d or standard PEI22K polyplexes. While prevention of endosomal acidification had no significant effect on transfection with MK-A1/1 polyplexes, transfections performed with BAA-2/1-3d complexes were seriously influenced. As it is known for PEI22K, which in this experiment responded to the bafilomycin treatment by a 30-fold decrease in transfection efficiency, a remarkable decrease could also be observed for the BAA

polymer (**Figure 16**). For none of the polymers tested the cell viability was affected significantly by bafilomycin.

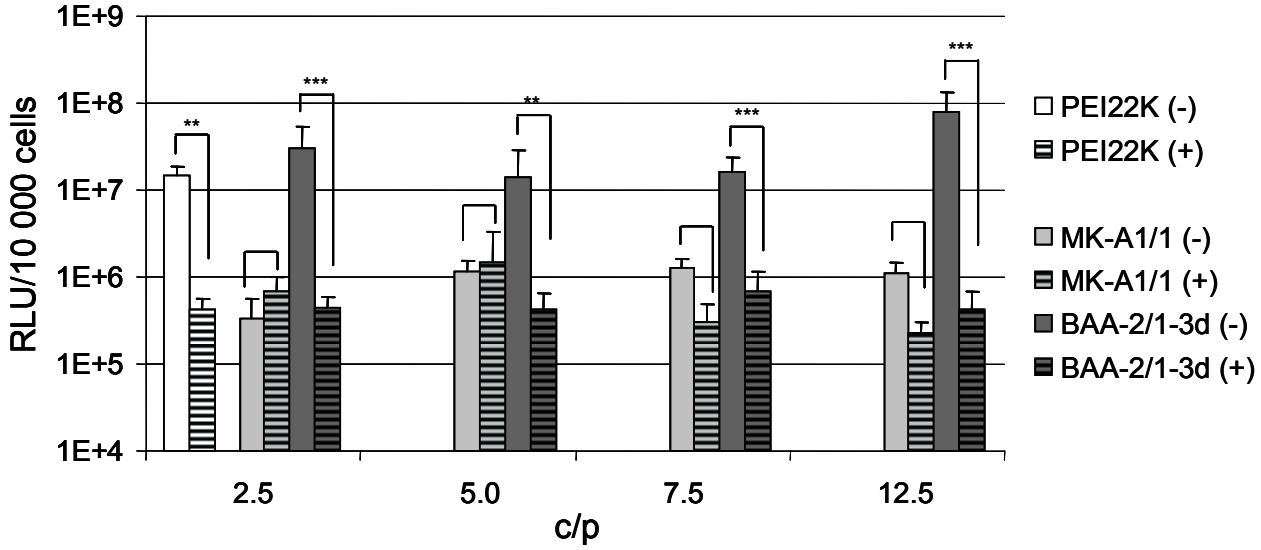


Figure 16. ***Influence of bafilomycin A1 on transfection efficiency of MK or BAA polymers in B16F10 cells.*** *PEI22K was applied at optimal N/P ratio of 6. The bars represent the mean of three independent experiments, each performed in triplicate. (-) indicates transfection in absence of bafilomycin A1, (+) stand for the presence of bafilomycin A1, 100 nM.*

3.2.1.4 In vivo *biocompatibility studies*

As the acid degradable polymers showed promising results concerning biocompatibility *in vitro*, we decided to examine their *in vivo* toxicity in the next step. Therefor different amounts of selected plain polymers (BAA-2/1-3d, MK-A1/1 or stable controls) were administered to 8-weeks old female Balb/c mice via tail vain injection. Acetal comprising polymers were well tolerated by the mice at amounts of 50 µg/20 g mouse, and no changes in liver tissue were detectable (**Figure 17,** upper two pictures on the left). Doubling the polymer amount to 100 µg/20 g mouse was still well tolerated for BAA-2/1-3d and no pathological alterations in the analyzed liver tissue were found; for MK-A1/1 this was also the case for two out of three mice, while one of the mice showed first slight signs of developing a fatty liver at the 100 µg dose. In contrast, stable BM-A1/1 and LT-OEI-HD polymers caused fatty livers already at 50 µg/20 g mouse (**Figure 17,** upper two pictures on the right) and the 100 µg dosage was lethal in case of BM-A1/1. For control polymer PEI25K the dosage had to be even reduced to 25 µg/20 g mouse, as all three animals died,

when treated with 50 µg. With the 25 µg PEI25K injection hepatocytes lost their normal shape indicating necrotic changes (**Figure 17**).

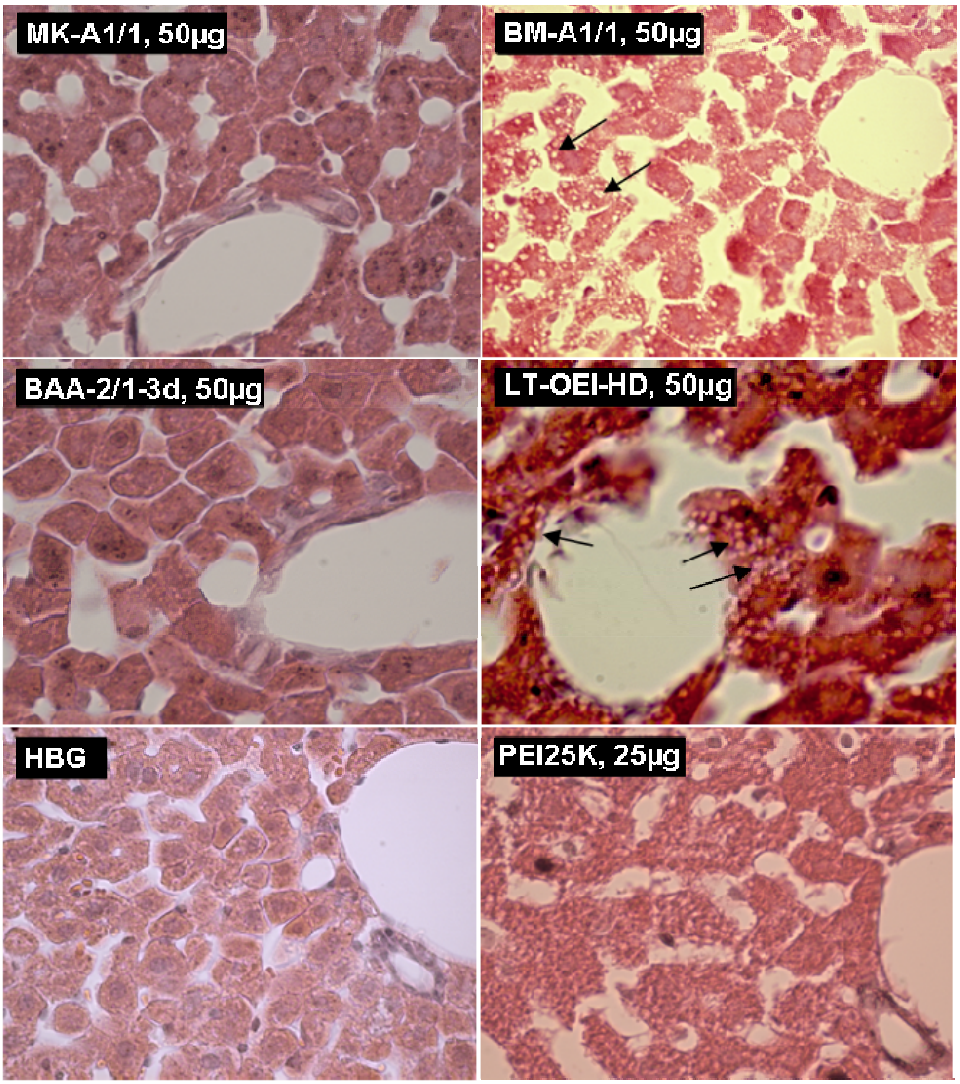


Figure 17. In vivo *study on toxicity of the free polymers in Balb/c mice. Histological examination on liver tissue (hematoxylin-eosin staining) 48 h after i.v. administration of indicated amounts of free polymer per 20 g mouse. Application of 50 µg of acid-labile polymers MK-A1/1 and BAA-2/1 induced no histopathological changes in the liver compared to HBG treated control animals. In contrast the same dosage of acid-stable BM-A1/1 and LT-OEI-HD polymers resulted in the development of fatty livers (black arrows indicate lipid droplets in hepatocytes after BM-A1/1 and LT-OEI-HD treatment). Mice treated with 25 µg of PEI25K even showed necrotic changes in liver tissue, which can be seen by the impaired morphology of hepatocytes in the lower right picture.*

3.3 Further optimization of OEI-MK polymer as gene carrier

The degradable MK and BAA polymers described below show an improved toxicity profile compared to their acid-stable counterparts and their transfection efficiency is promising; however, transfection properties might be improved further. Thus, the acid-degradable MK-polymer was chosen as an auspicious starting point for the development of degradable polymers with enhanced transfection efficiency. For the change and optimization of the polymer`s physicochemical properties, attention was turned on four aspects:

Polymer size – as already mentioned, it is well known that an increased molecular weight often comes along with enhanced transfection efficiency of polymers (*33, 58, 59*). Further, low polydispersity indices (PDI) are preferred over widespread size distributions, as characteristics of polymers with low PDI are better defined. Both parameters can be improved for MK-A1/1.

Amine density – beside the molecular mass also the amine density of a polycation has an influence on its gene transfer properties. High amine densities, as found for example in PEI, are associated with a high buffering capacity, facilitating endosomal release (*58*) and leading to high transfection efficiency.

Hydrophilic shielding – the introduction of neutral hydrophilic PEG-chains into gene vectors does not only reduce vector toxicity but – in certain cases – can also improve the gene transfer efficiency (*88-90*).

Hydrophobicity – viruses have been shown to utilize hydrophobic, membrane lytic domains to induce endosomal release (*24*). Equipping polymers with hydrophobic molecules could therefore also help to facilitate their endosomal release.

3.3.1 Polymers with increased size and amine density

3.3.1.1 Increased molecular weight: MK-A1/1-Fr.I, MK-A1.2/1, MK-A1.5/1

a) Polymer syntheses and analysis of their biophysical properties

As described above, acid-degradable MK-A1/1 polymer is synthesized by reacting OEI800 in a 1/1 ratio with the MK-linker, followed by dialysis using a 3.5 kDa cut off membrane. In order to obtain polymers with higher m.w., the 1/1 linker/OEI800 input ratio as well as the dialysis cut off were increased. Raising the ratio up to 1.2/1 or 1.5/1 resulted in products containing 1.05 or 1.23 linkers per OEI800 respectively; for

comparison: MK-A1/1 contained only 0.93 linkers per OEI. This, combined with the use of a 6-8 kDa m.w. cut off dialysis membrane for purification, yielded MK-A1.2/1 (red solid, 51 % yield) and MK-A1.5/1 (red solid, 60 % yield) as polymers with enhanced average molecular mass compared to MK-A1/1. The increase in m.w. was attested by SEC using Superdex 75 material for the investigation of the low m.w. range and Superdex 200 for separations in the high m.w. range. On Superdex 75 a decrease in the low m.w. fractions (oligomers of about 5 kDa and less) was detected for MK-A1.2/1 and MK-A1.5/1 compared to MK-A1/1 (**Figure 18**), while the elution profiles on the Superdex 200 column showed an increase in the high m.w. range (polymers with m.w. of 25 – 50 kDa) for MK-A1.2/1 and 1.5/1 (data not shown). SEC also revealed that all polymers possessed a high polydispersity and residual OEI800 monomers were still present in the dialyzed products. Thus, another approach towards higher average m.w. – and ideally also towards a lower polydispersity – was the fractionation of MK-A1/1. Therefor the polymer was separated on a Superdex 75 column into three fractions, starting with MK-A1/1-Fr.I (**Figure 18**). This fraction comprised the polymer-portion eluting between minute 9 and 15, with the highest m.w. parts reaching sizes of about 50 kDa; mono- and oligomers up to about 5 kDa were removed. This fraction I was followed by MK-A1/1-Fr.II, which ranged from minute 16 to 20 and contained oligomers of about 2-5 kDa in size. Finally MK-A1/1-Fr.III was collected, containing monomers eluting after 21 minutes and later. Polymer concentrations in the collected fractions were determined by copper complex assay.

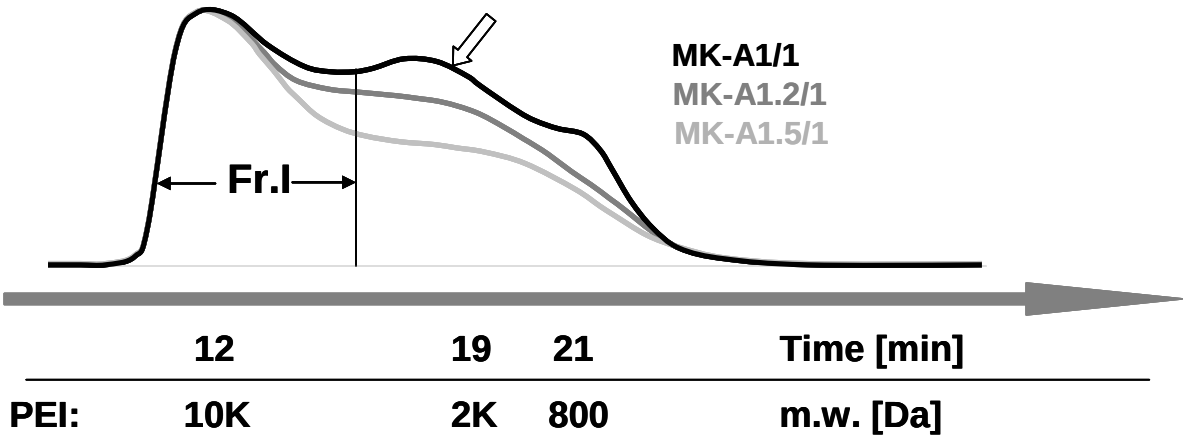


Figure 18. ***Polymer molecular weight characterization and fractionation via SEC:*** *elution profiles of MK-A1/1 (black line), MK-A1.2/1 (dark grey line), MK-A1.5/1*

(light grey line) on a Superdex 75 column. Decrease of low molecular weight fractions in MK-A1.2/1 and MK-A1.5/1 is marked by the white arrow.
Numbers on the scale below the curves indicate the elution times and sizes of standard PEI polymers with known molecular weight.

b) *In vitro* transfection results (luciferase reporter gene expression) and metabolic activity of transfected cells

Transfection efficiency as well as polyplex toxicity of the polymers with increased m.w. was evaluated *in vitro* on B16F10 and Neuro2A cells. The relation between polymer size and efficiency of gene transfer became clearly visible when MK-A1/1 was compared to its three fractions with different molecular masses. MK-A1/1-Fr.I, which is composed of the highest m.w. portion of MK-A1/1, showed transfection results clearly superior not only to the lower m.w. fractions but also to the non-fractionated polymer on both cell lines (**Figure 19A, C**). While transfection efficiency rose from Fr.II and native MK-A1/1 to Fr.I, toxicity did not increase in parallel, and Fr.I showed the same high biocompatibility as MK-A1/1 or Fr.II. The same effect of enhanced transfection efficiency without increased toxicity was observed for MK-A polymers that were directly synthesized with increased m.w. (MK-A1.2/1 and MK-A1.5/1). In cell culture experiments MK-A1.2/1 and MK-A1.5/1 showed results similar to MK-A1/1-Fr.I, exceeding native MK-A1/1 but not MK-A1/1-Fr.I (data not shown). Taken together, shifting the size of MK-A towards the higher m.w. range improved the polymer´s properties as gene vector.

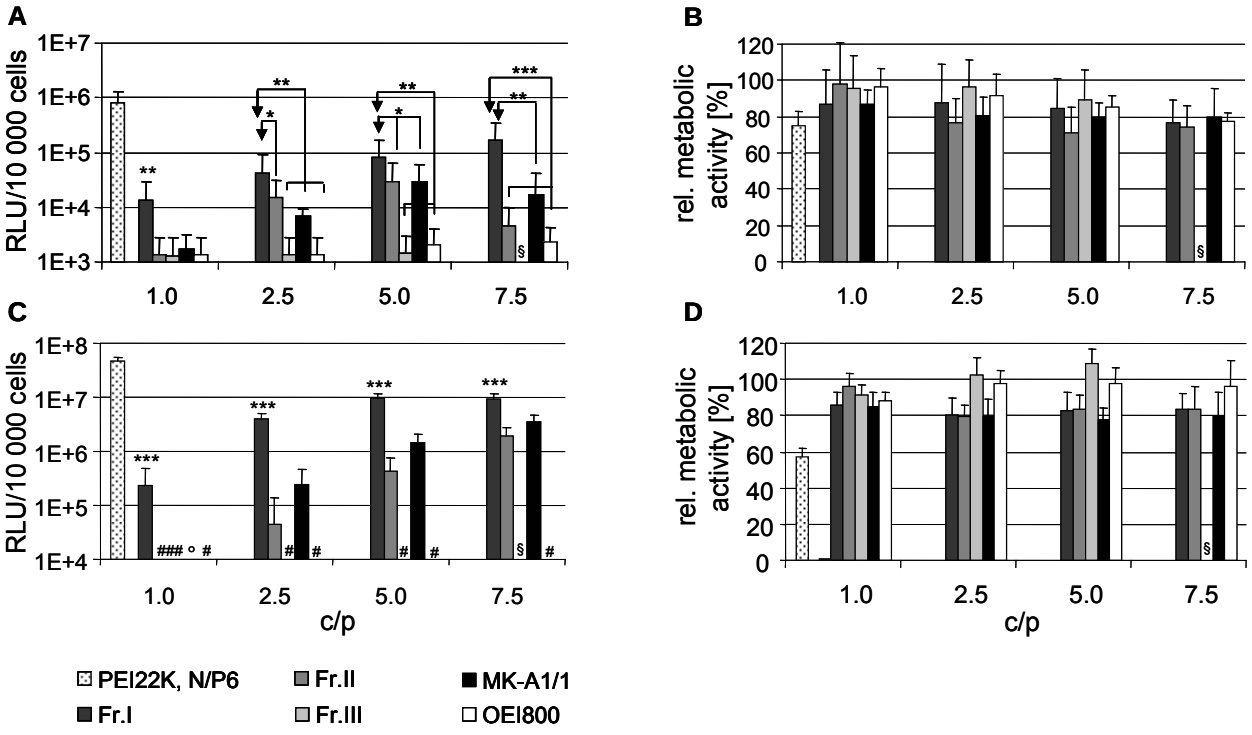


Figure 19. In vitro *transfection experiments with fractionated MK-A1/1:* *influence of molecular weight on in transfection efficiency (luciferase assay,* **A** *and* **C***) and metabolic activity (MTT assay,* **B** *and* **D***) in Neuro2A (upper figures) and B16F10 cells (lower figures).*
Each bar represents the mean ± sd of three individual experiments, each performed in triplicate. Significances were calculated for Fr.I values in comparison to MK-A1/1, Fr.II, Fr.III and OEI800. § not determined; # values >350 and <550 RLU/10 000 cells; ° 1250 RLU/10 000 cells. Statistics given in figure 19C refer to the comparison of Fr.I with Fr.II, Fr.III, MK-A1/1 and OEI800.

3.3.1.2 Increased amine density: BM-B1/1, MK-B1/1

a) Syntheses, chemical and biophysical analysis

Apart from the size of the polymer itself, also the size of its monomer units can be changed. Utilization of larger monomers, for instance, leads to an enhancement of the amine density in the resulting polymer.

BM-B1/1 and MK-B1/1 were therefore synthesized following the protocol for BM-A1/1 or MK-A1/1 respectively, replacing OEI800 starting material by the larger PEI1.8K. Within the purification, the 3.5 kDa dialysis membrane was replaced by a 6-8 kDa cut off membrane. Freeze drying of the dialyzed products finally resulted in red-colored solids with yields of 68 % and 43 % in case of BM-B1/1 and MK-B1/1, respectively. According to SEC analysis on Superdex 75 and 200 columns, sizes of BM-B1/1 and MK-B1/1 polymers resembled to each other. Compared to the polymer sizes

determined for MK-A1/1 and BM-A1/1 they were shifted towards the higher m.w. range: the fraction of oligomeric sized components decreased in favour of the high m.w. fraction of ≥ 40 kDa. A peak maximum appeared around 10 kDa, another one at ≥ 50 kDa. To ensure validity of the data obtained by SEC on Superdex material, the acid-stable BM-B1/1 polymer was additionally analyzed on a Novema 300 column. As already mentioned, this system requires an acidic mobile phase (pH 2.8) and is therefore not suitable for the analysis of acid-labile MK-B1/1. The analysis of BM-B1/1 resulted in Mn = 11.1 kDa and Mw = 108 kDa with a polydispersity of 9.8. For comparison: for BM-A1/1 we found Mn = 8.5 kDa and Mw = 36 kDa with a polydispersity of 4.2. These results confirmed the findings obtained by size exclusion on Superdex 75 and 200.

To investigate DNA binding affinity of the PEI1.8K based polymers an EtBr exclusion assay was performed. Already PEI1.8K starting material itself bound to DNA stronger than the smaller OEI800. DNA compacting properties of the derived polymerization products MK-B1/1 and BM-B1/1 were equivalent to those of PEI1.8K in the EtBr exclusion assay (**Figure 20**) and only marginally stronger than those of the A-line polymers.

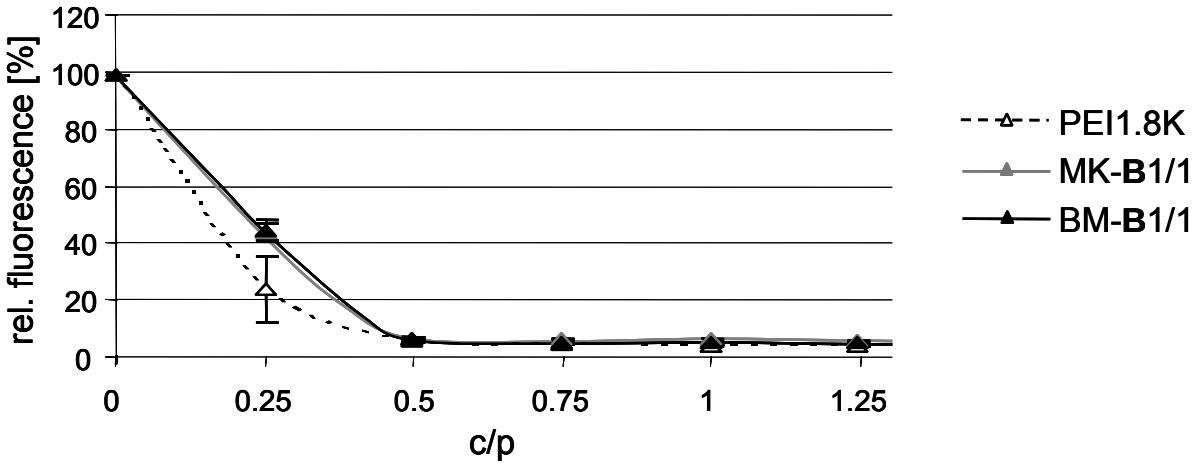


Figure 20. ***Ethidium bromide exclusion assay in HBG, pH 7.4.*** *DNA binding affinity of the B-line: BM-B1/1, MK-B1/1 and PEI1.8K. Each point represents the mean ± sd of three experiments.*

b) ***In vitro*** **transfection results (luciferase reporter gene expression) and metabolic activity of transfected cells**

Transfection experiments with BM-B1/1 and MK-B1/1 in B16F10 cells showed improved *in vitro* transfection efficiency for the polymers with enhanced amine density compared to the corresponding OEI800 based counterparts (**Figure 21**). Again, an additional fractionation of MK-B1/1 influenced the transfection results favorably, but not to the same extend as it was the case for MK-A1/1. At a c/p ratio of 2.5 – the optimal ratio for MK-B1/1 and MK-B1/1-Fr.I – these both polymers reached transfection levels in the range of the golden standard PEI22K at its optimal N/P ratio of 6. However, the improved transfection efficiency of MK-B polymers over MK-A was paid to some extend at the costs of an increased toxicity; thus, cell viability after treatment with MK-B1/1 (c/p ratio of 2.5) corresponded to that after treatment with PEI22K (N/P 6) (75 % and 73 % relative metabolic activity, respectively), or was only slightly higher (82 %) in case of MK-B1/1-Fr.I.

Stable control polymer BM-B1/1 showed about the same transfection efficiency as its acid-labile analog MK-B1/1, but became more toxic at higher c/p ratios (≥ 5).

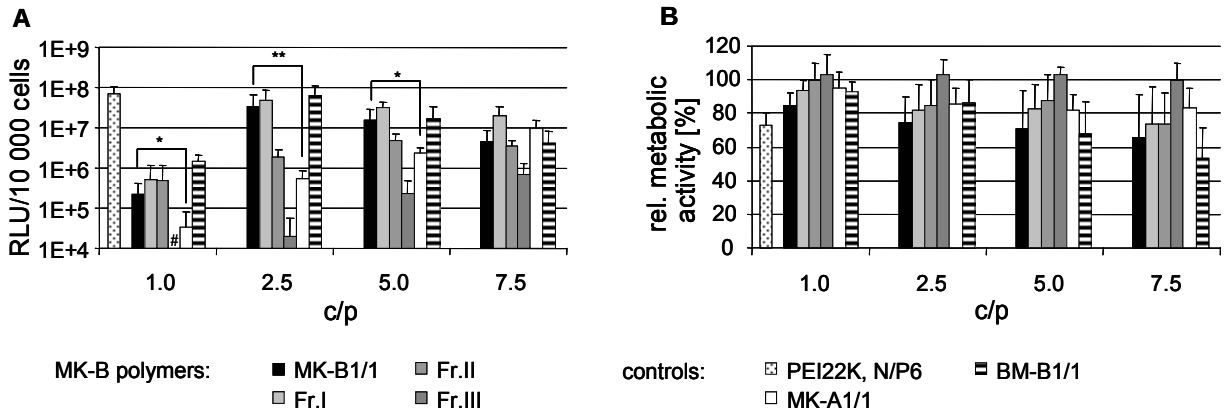
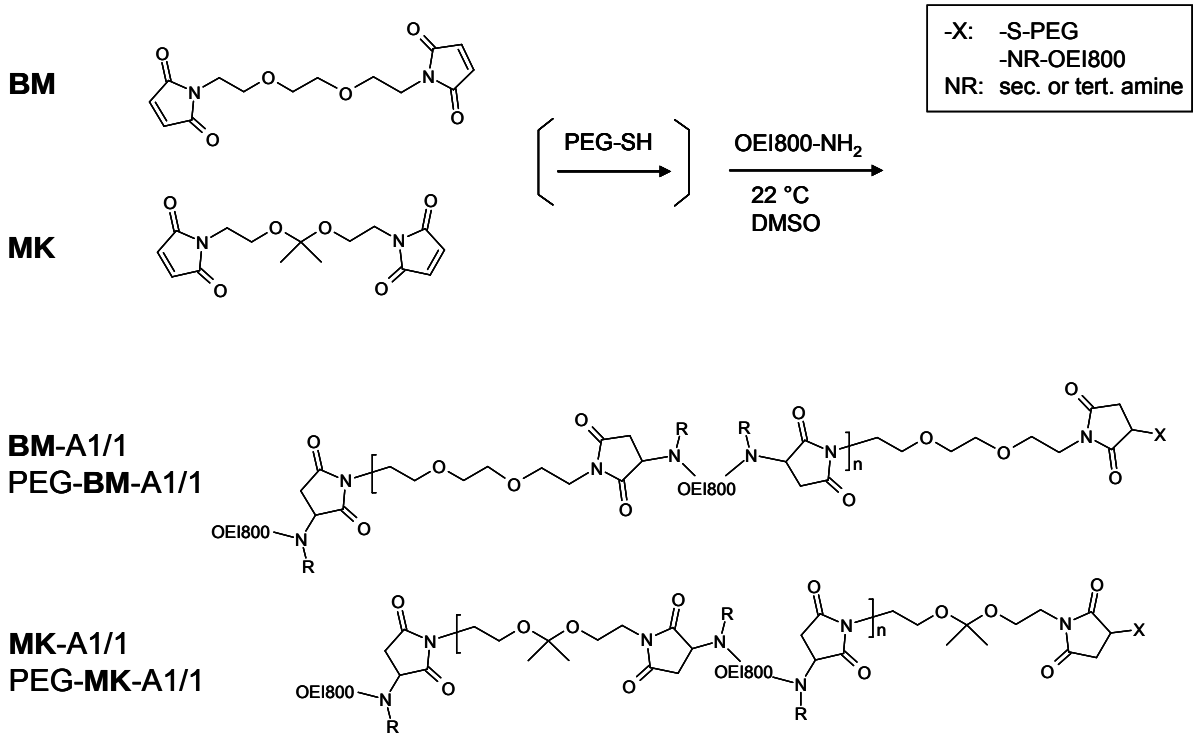


Figure 21. ***Evaluation of the* in vitro *transfection efficiency (luciferase assay, A) and toxicity (MTT assay, B) of MK-B1/1 (non-fractionated and fractionated) and BM-B1/1 polyplexes compared to MK-A1/1 in B16F10 cells.*** *Each bar represents the mean ± sd of three individual experiments, each performed in triplicate. # 1.7 × 10^3 RLU/10^3 cells. Statistics were calculated for the differences in transfection efficiency of MK-B1/1 and MK-A1/1.*

3.3.2 PEGylated polymers: PEG-BM-A1/1 and PEG-MK-A1/1

a) Syntheses and biophysical analysis



Scheme 5. ***Syntheses of MK-A1/1, BM-A1/1 and their PEGylated variants PEG-MK-A1/1 and PEG-BM-A1/1.*** *In case of the PEGylated polymers, the linker is reacted first in 19-fold molar excess with PEG-SH before OEI is added.*

To introduce PEG into MK-A1/1 or BM-A1/1 (**Scheme 5**) 5 % (n/n) of the OEI800 were replaced by thiol modified PEG of 5 kDa (PEG-SH). The purified and dried polymers appeared as pink cotton-like voluminous solids. PEG content as well as linker to OEI ratios in the resulting polymers were determined by ^{1}H NMR analysis: for PEG-BM-A1/1 a proportion of 0.05/1.3/1 PEG/linker/OEI800 was found, while this ratio was 0.08/1.09/1 in case of PEG-MK-A1/1. An EtBr exclusion assay was performed in order to investigate if PEGylation hampers DNA binding capacity of the polymers. In this assay the presence of PEG caused no (c/p ratios ≥ 0.5) or only marginal (c/p < 0.5) impairment of DNA binding (**Figure 22**).

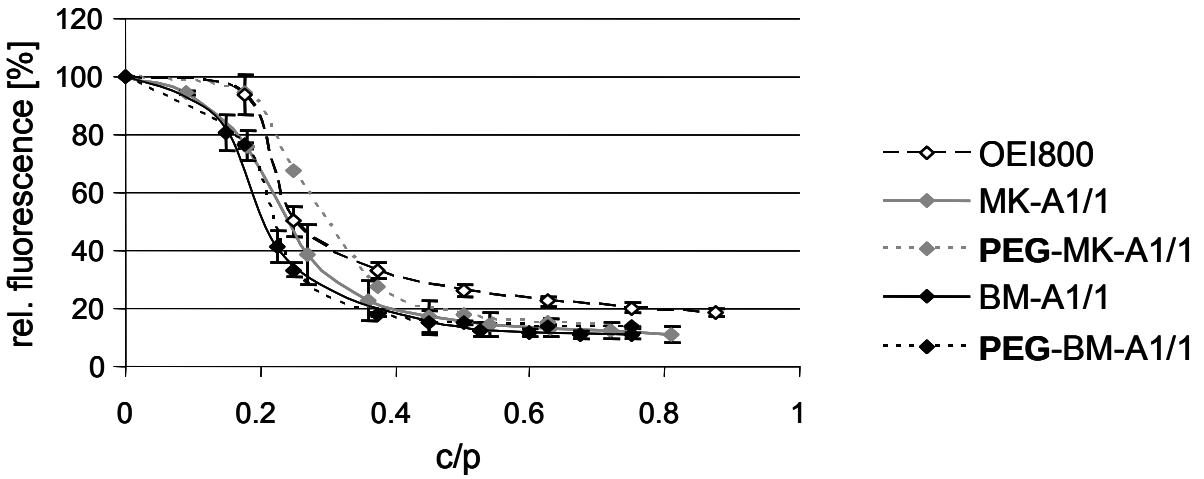


Figure 22. ***Ethidium bromide exclusion assay (HBG, pH 7.4).*** *Influence of PEGylation on DNA binding: PEG does not or only slightly (at very low c/p ratios) hamper binding ability of the polymers. Each point represents the mean ± sd of three experiments.*

Next, the effect of PEG shielding on polyplex size and surface charge was investigated. Therefor, MK-A1/1 and BM-A1/1 conjugates with or without PEG were mixed in HBG with pDNA at several c/p ratios. Differences in particle size as well as in zeta potential were found for the PEGylated particles compared to the PEG-free ones. Upgraded particle stabilization upon PEGylation was noticeable especially in the low c/p range, where particles are compacted only loosely: while non-PEGylated polyplexes interacted with each other, showing rather big sizes at lower c/p ratios, PEGylated particles possessed constant small sizes around 100 nm independent from the c/p ratio (**Figure 23A**). The shielding effect of PEG became even more apparent when particles were analyzed for their surface charge. Here, PEGylated particles showed almost neutral zeta potentials over the whole c/p range tested, whereas surface charges of non-PEGylated polyplexes depended strongly on the c/p ratio and reached from negative values at c/p of 0.25 up to about +25 mV for c/p of 4 or higher (**Figure 23B**). This study demonstrates that PEGylation of MK- and BM-A1/1 polymers has been conducted successfully and the PEG content chosen is sufficient to achieve total shielding of MK-A1/1 and BM-A1/1 polyplexes.

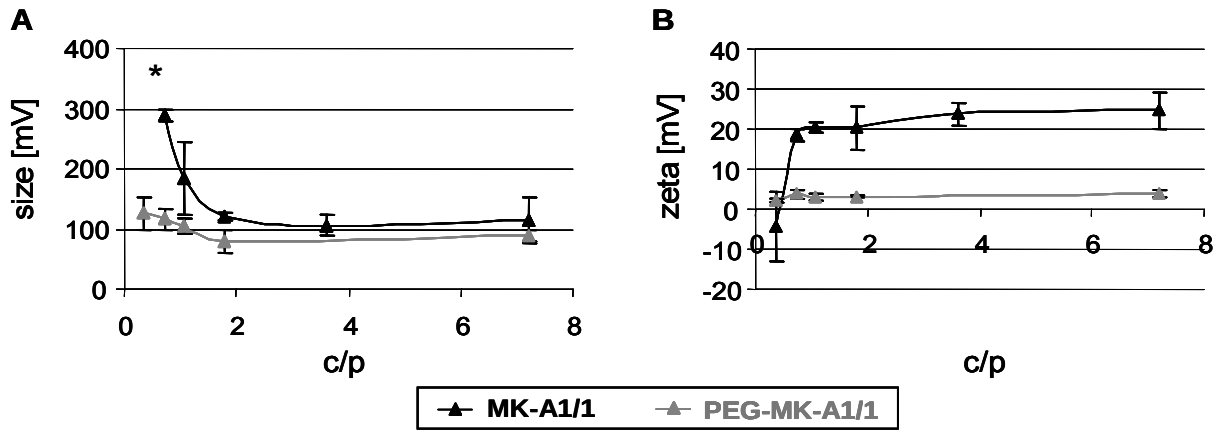


Figure 23. ***Determination of particle size (A) and surface charge (B) in presence or absence of PEG in HBG pH 7.4.*** *Particles were either formed with MK-A1/1 (black triangles) or PEG-MK-A1/1 (grey triangles) and pDNA at different c/p ratios. Nearly neutral zeta potentials and stable small particle sizes of the PEGylated particles demonstrate the shielding effect of PEG-MK-A1/1 compared to non-PEGylated MK-A1/1. Each point represents the mean ± sd of three experiments.*
** aggregation (particles > 1 nm) at c/p ratios < 0.5.*

For the introduction of PEG into the polymers the same linkage (MK- or BM-linker respectively) was used as for the polymerization process of the polymer itself; therefore, in PEG-MK-A1/1 the PEG moiety is attached reversibly via the acetal linkage, while in PEG-BM-A1/1 the linkage is irreversible. As this effects polyplex shielding behaviour, shielding kinetics were examined in a further study. Polyplexes were prepared in HBG at c/p 1 and diluted either with buffer of pH 7.4 or with buffer of pH 5.0. The samples were then incubated at 37 °C for different time periods and changes in particle size and surface charge were analyzed. While PEG-MK-A1/1 particles stayed stable at pH 7.4, in acidic medium these particles grew in size and finally aggregated; in parallel their zeta potential increased over time (**Figure 24A**). These data demonstrate that maleimido-acetal-linked PEG allows shielding in physiological fluids but at the same time is acid-reversible, which is of interest for endosomal deshielding of polyplexes. In contrast PEG-BM-A1/1 particles changed their characteristics neither at physiologic nor at acidic pH, which attests that PEGylation is stable in this case, independent from the existing pH (**Figure 24B**).

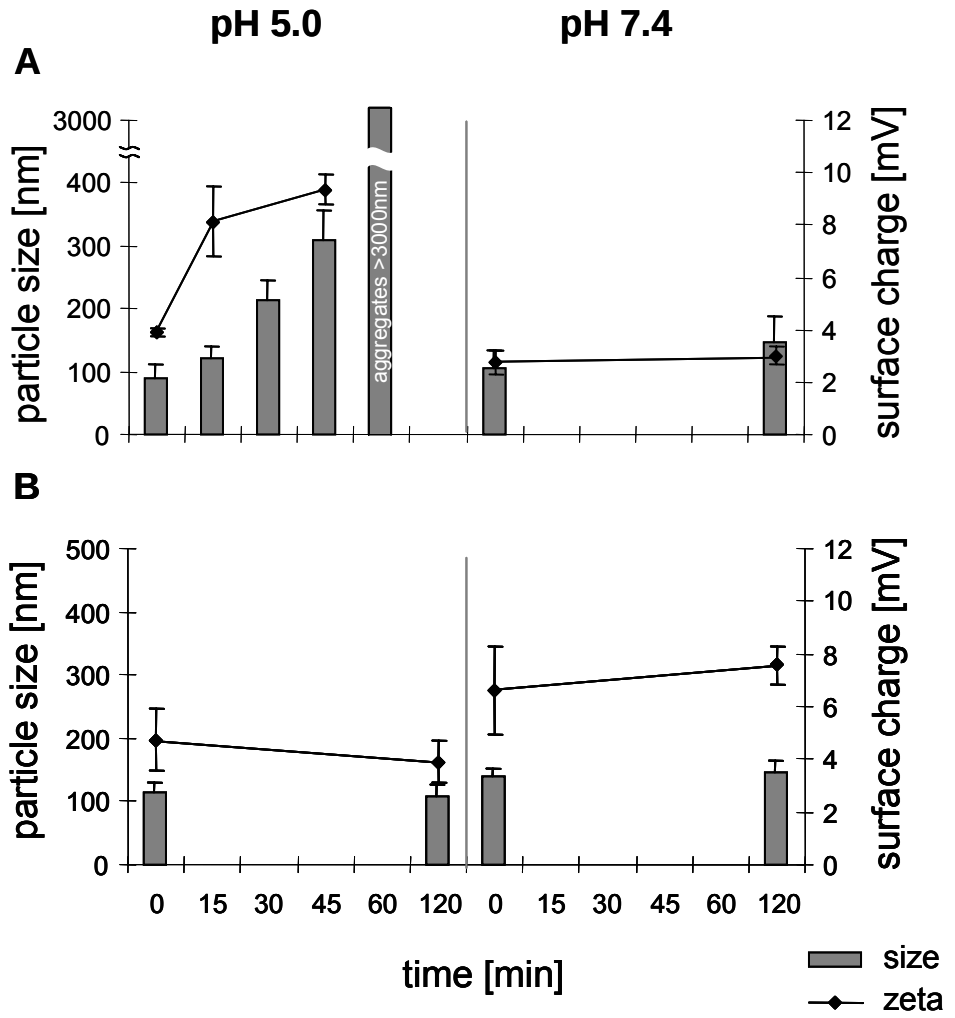


Figure 24. ***Particle size and surface charge of PEG-MK-A1/1 (A) and PEG-BM-A1/1 (B) polyplexes (c/p = 1) measured at different time points after incubation at 37 °C.*** *Measurements were performed either in 20 mM NaOAc pH 5.0 containing 75 mM NaCl (left panels) or in 20 mM Hepes pH 7.4 containing 75 mM NaCl (right panels). Reversible PEGylation at pH 5.0 in case of PEG-MK-A1/1* (**A**, left panel). *No removal of PEG from PEG-MK-A1/1 particles at physiologic pH* (**A**, right panel). *Polyplexes made of non-degradable PEG-BM-A1/1 stably shielded at both pHs* (**B**). *Each point or bar represents the mean ± sd of three experiments.*

b) In vitro transfection results (luciferase reporter gene expression, enhanced green fluorescent protein expression) and metabolic activity of transfected cells

The effect of PEGylation of MK-A1/1 on polyplex efficiency in gene transfer was evaluated in B16F10 cells (**Figure 25**). Thereby PEG-MK-A1/1 was compared to non-PEGylated MK-A1/1 and to the stable analogs BM-A1/1 and PEG-BM-A1/1. In both cases – MK as well as BM polymer – PEGylation improved compatibility of the polyplexes in B16F10 cells significantly, especially at higher c/p ratios (**Figure 25A**).

PEG-MK-A1/1, basing on the degradable MK-A1/1 polymer, was thereby significantly less toxic than corresponding nondegradable PEG-BM-A1/1. Furthermore, the experiments demonstrated that not only PEGylation per se but also the kind of PEG linkage used (stable, labile), has an impact on transfection results.

In terms of transfection efficiency (luciferase assay) PEGylation had a negative effect on the BM-linked polymer, thus reducing gene expression remarkably (**Figure 25B**). For MK-A1/1 in contrast, the reversible PEGylation improved transfection efficiency of the polymer; at high c/p ratios (12.5) PEG-MK-A1/1 even showed gene expression values comparable to those reached with PEI22K. At c/p ratios of 10 and 12.5 the superiority of PEG-MK-A1/1 was significant over all three other polymers (MK-A1/1, BM-A1/1 and PEG-MK-A1/1).

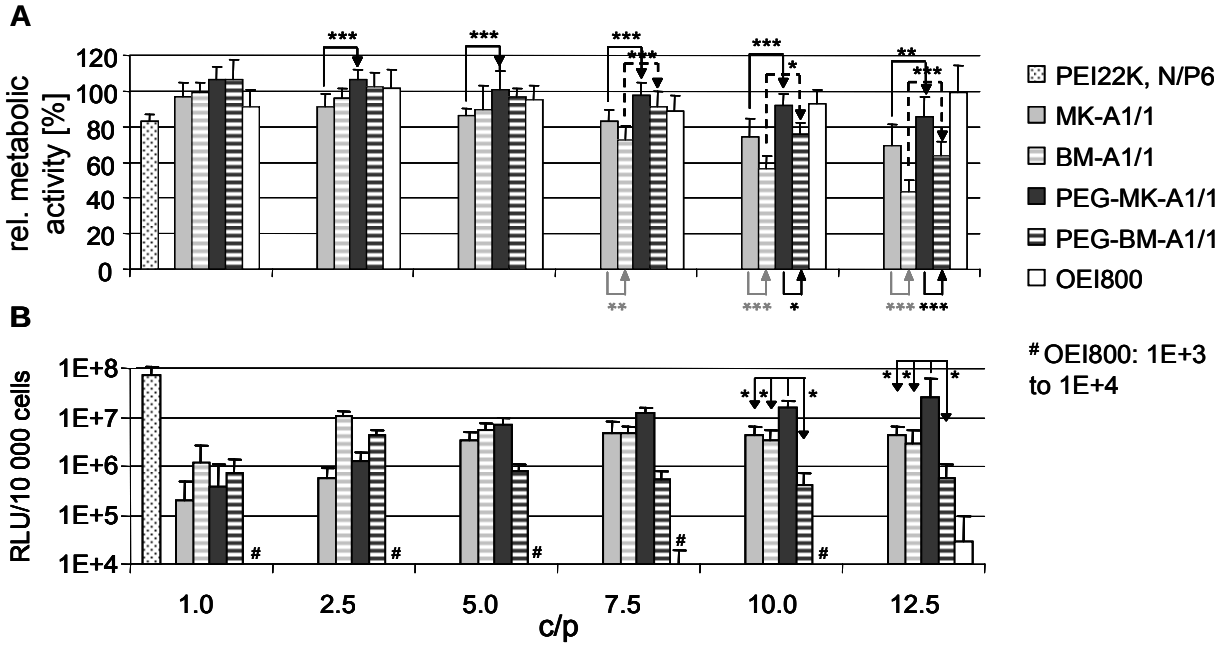


Figure 25. ***Influence of PEGylation of MK-A1/1 and BM-A1/1 on transfection efficiency and polyplex toxicity in B16F10 cells.*** *While stable PEGylation (PEG-BM-A1/1) only improves polyplex compatibility but decreases transfection efficiency, acid-reversible PEGylation has positive effects on both features. The bars in the diagrams show the mean ± sd out of three individually performed experiments, each of them performed in triplicate.*

To further elucidate the differences between the four polymers, FACS analysis of pEGFP transfected B16F10 cells was performed (**Figure 26**). PEI22K polyplexes were used again at their optimal 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-BM-A1/1, PEG-MK-A1/1, MK-A1/1 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. Therefor, in contrast to the luciferase assay, the higher toxicity of BM polymers is not represented, which makes toxic polymers appear in a much better light. It is hence not reasonable to compare the results of toxic BM-linked polymers directly with those obtained for highly compatible MK-linked ones. Nevertheless, the study allows to gain some insight into the effects of PEGylation on the transfection properties of MK-A1/1 or BM-A1/1. Regarding the mean fluorescence intensity (MFI) per cell, stable PEGylation of BM-A1/1 resulted in a loss of reporter gene expression also in this experiment: while for BM-A1/1 a MFI of 666 was measured, for PEG-BM-A1/1 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 MK-A1/1 heightened the MFI-value from 466 (MK-A1/1) to 600 (PEG-MK-A1/1) and no decrease in the number of transfected cells could be observed. Taken together, this study confirmed that transfection properties are affected positively by acid-labile PEGylation, while they worsen in the presence of stable PEG.

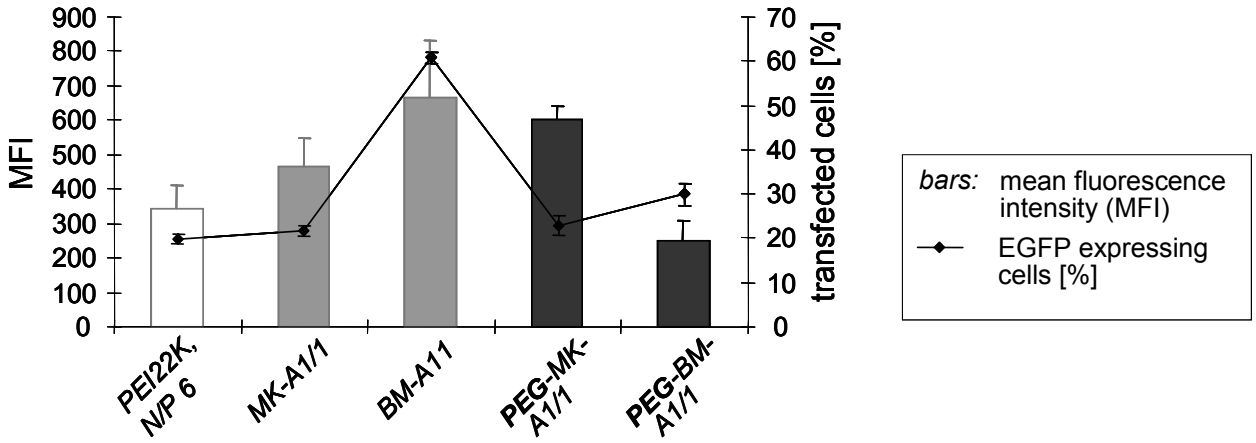


Figure 26. ***Enhanced green fluorescent protein (EGFP) gene expression in B16F10 cells.*** *Polyplexes were prepared at c/p ratio of 10 (for PEI22K at N/P = 6) in HBG. The diagram shows a representative experiment, each point or bar demonstrates the mean of a triplicate. (MFI: mean fluorescence intensity).*

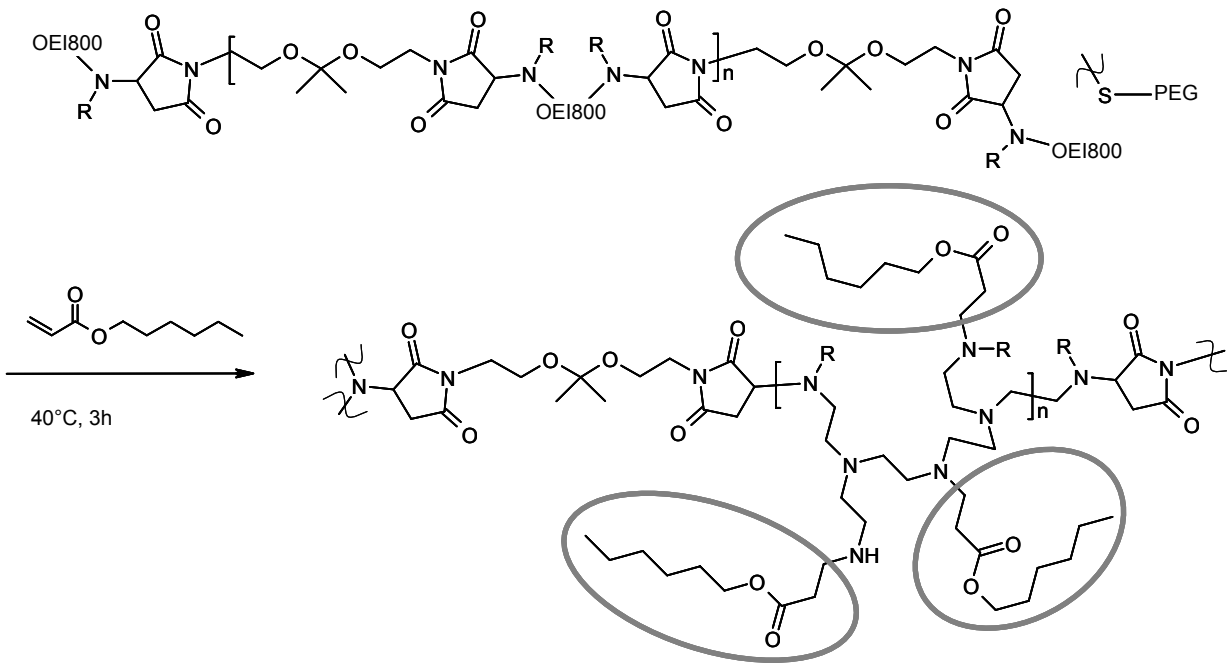
3.3.3 Hexyl acrylate modification of MK-A1/1 and PEG-MK-A1/1

a) Syntheses and biophysical analysis

Six different polymers equipped with hydrophobic hexyl acrylate (HA) domains were synthesized: HA2.5-MK-A1/1, HA5-MK-A1/1, HA10-MK-A1/1 and HA2.5-PEG-MK-A1/1, HA5-PEG-MK-A1/1, HA10-PEG-MK-A1/1. The nomenclatur of the polymers refers to the HA/OEI input ratios used (i.e. HA2.5 indicates an HA/OEI800 ratio of 2.5/1) and to the polymer prototype the polymers are derived from (MK-A1/1 or PEG-MK-A1/1).

The initial step in the production of HA-polymers was the synthesis of MK-A1/1 or PEG-MK-A1/1. Instead of working up, the respective amounts of hexyl acrylate (HA/OEI input ratios: 2.5/1 for HA2.5 polymers, 5/1 for HA5 polymers and 10/1 for HA10 polymers) were added directly to the reaction mixtures of MK-A1/1 or PEG-MK-A1/1 and the reaction was continued for another 3 h at elevated temperature (**Scheme 6**). The subsequent steps (dialysis, freeze drying) were performed as described for MK-A1/1. Depending on the amount of HA the resulting products differed in their physical properties: HA10 variants possessed viscous consistency, were transparent and of red to auburn colour; HA5 variants were also dark red or auburn but of much higher viscosity, nearly solid; HA2.5 polymers finally were pink and exhibited a voluminous cotton-like structure. In general, the PEGylated polymers showed darker colours than their non-PEGylated variants. While HA2.5 and HA5 polymers could be dissolved in water quite easily, HA 10 polymers were soluble only at low concentrations of about 2 mg/mL and less.

EtBr exclusion assay performed with all HA polymers indicated that HA does not interfere with the DNA binding of the polymers up to HA/OEI ratios of 5. For HA10-MK-A1/1 in contrast – and even more for HA10-PEG-MK-A1/1 – DNA condensing ability was reduced remarkably. Measurements on particle size also suggested that polyplexes were more stable and compact, the less HA they contained; this is in accordance with the results from EtBr exclusion assay. Further, it was found that HA mediated no shielding effect and only additionally PEGylated particles exhibit neutral surface charges.



Scheme 6. ***Schematic illustration of the HA-modification of MK-A1/1 or PEG-MK-A1/1.*** *Hydrophobic hexyl acrylate residues are reacted onto MK-A1/1 or PEG-MK-A1/1 polymers. R = H or oligoethylenimine units; hydrophobic HA moieties in the polymer are encircled in grey.*

b) *In vitro* transfection results (luciferase reporter gene expression) and metabolic activity of transfected cells

The hydrophobic HA-MK and the amphiphilic HA-PEG-MK polymers were tested for gene transfer efficacy and toxicity on B16F10 and Neuro2A cells. Generally, all HA containing polymers showed increased toxicity compared to HA-free ones, with a direct correlation between HA amount and toxicity (**Figure 27B**). Interestingly, on B16F10 cells the PEGylated variants were even less compatible than the non-PEGylated HA polymers.

For some of the polymers transfection efficiency was enhanced compared to unmodified MK-A1/1. B16F10 cells for example showed highest gene expression up to 1 log unit below PEI22K with the both HA2.5 polymer variants. In contrast, in Neuro2A cells the highest transgene expression was obtained with HA10 variants and exceeded even the PEI22K standard (**Figure 27A**). HA5 modifications, however, were inefficient in both cell lines. Taking into account that – in case of high transfection efficiencies of HA polymers – the good biocompatibility of the original MK-A1/1 or PEG-MK-A1/1 polymers is completely deleted by the introduction of

hydrophobic HA moieties, the overall transfection efficiency/toxicity relation of HA-MK-A1/1 and HA-PEG-MK-A1/1 shows no advantage over the original polymers.

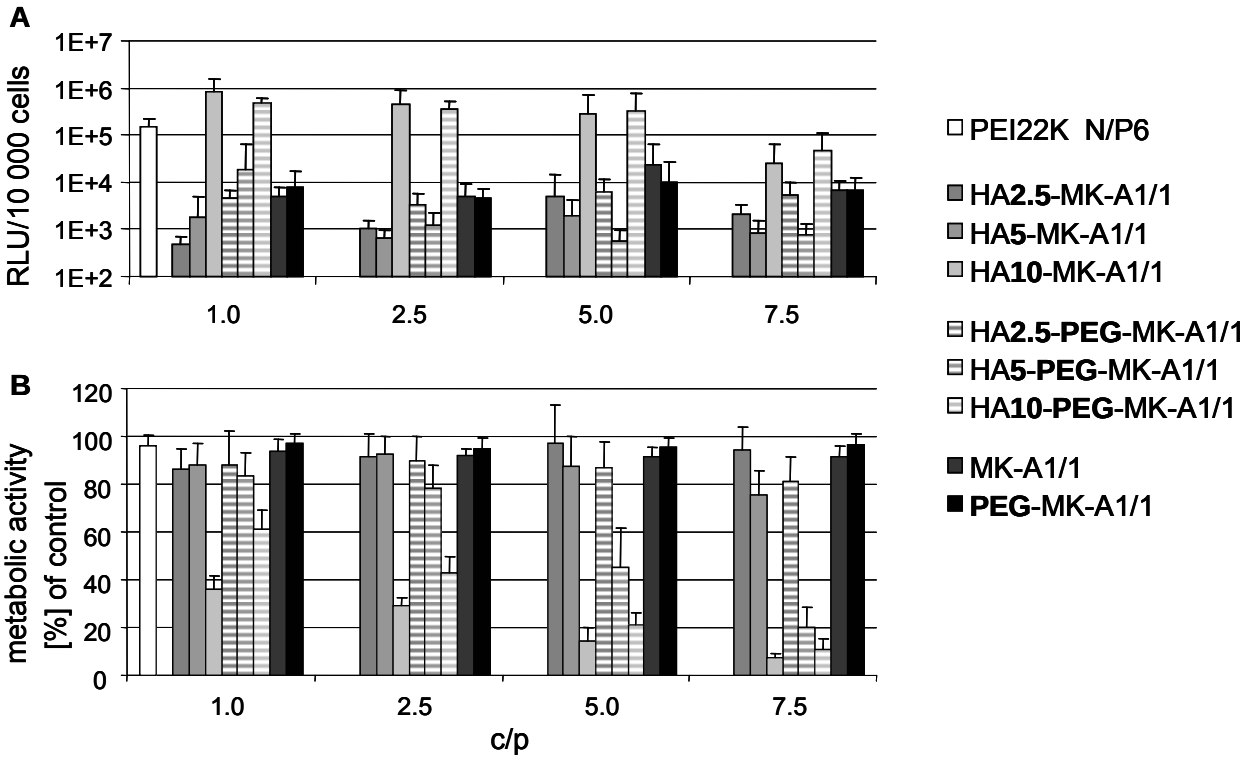
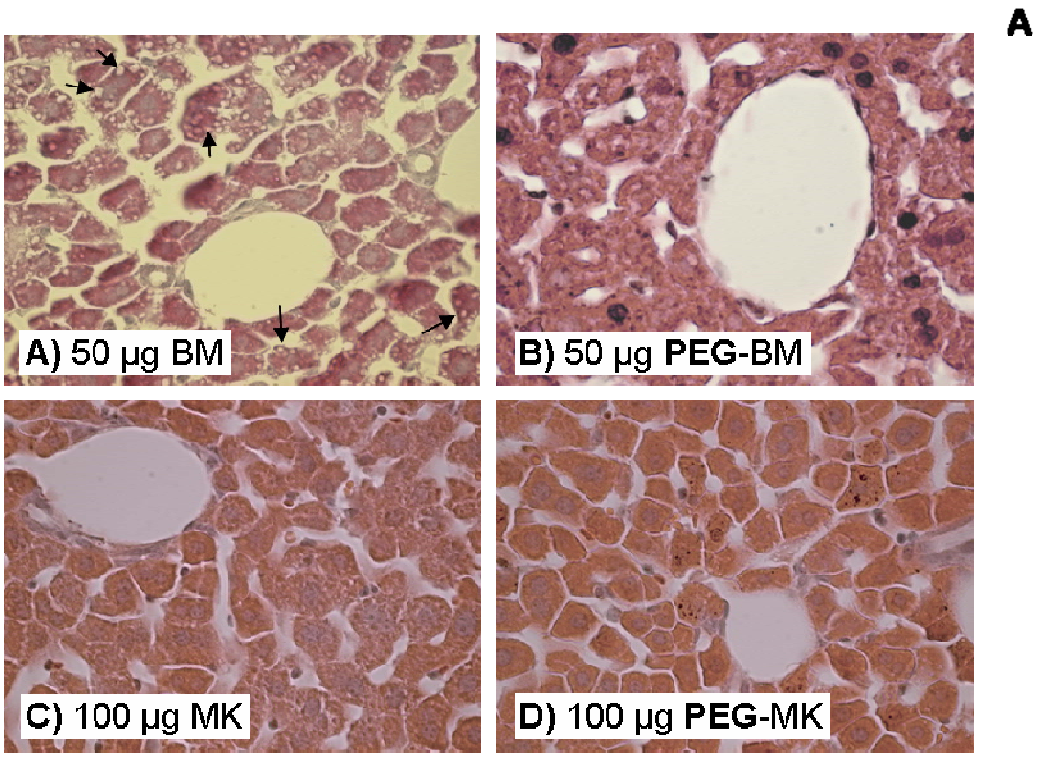


Figure 27. ***Influence of hydrophobization of MK-A1/1 and PEG-MK-A1/1 polymers on transfection efficiency and toxicity in Neuro2A cells.*** *While HA-modification in part could boost transgene expression, it also caused severe toxicity when used at high concentrations.*
Each bar represents the mean ± sd of three individual experiments each performed in triplicate.

3.3.4 *In vivo* biocompatibility studies of PEGylated polymers

The strategy of acid-sensitive PEGylation seemed to be the most promising concept concerning both, improved transfection efficiency and reduced polymer toxicity. Therefore, PEGylated MK and BM polymers were selected for further investigations on *in vivo* biocompatibility in mice. HBG-solutions of PEG-MK-A1/1 and its comparative polymers BM-A1/1, PEG-BM-A1/1 and MK-A1/1 were administered to 8-weeks old female Balb/c mice via tail vain injection. As mentioned previously, treatment with 100 µg polymer/20 g mouse is lethal in case of BM-A1/1. Dose reduction to 50 µg still induced the development of a fatty liver (**Figure 28A**, upper left picture A). PEGylation of the polymer (PEG-BM-A1/1) resulted in an improved biocompatibility: the dosage of 50 µg/20 g mouse was well tolerated by all three

animals and no pathological changes were observed in liver tissue (**Figure 28A**, upper right picture B). However, treatment with 100 µg of PEG-BM-A1/1 was survived only by one mouse out of three. This indicates that despite PEGylation



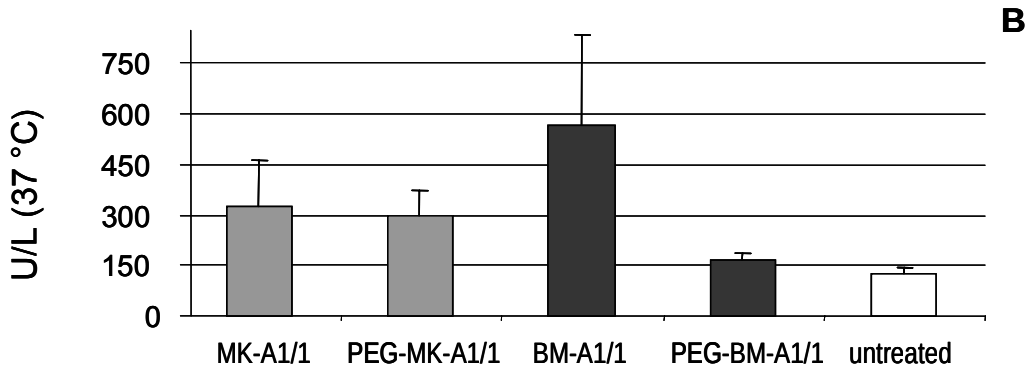


Figure 28. In vivo *study on toxicity of the free polymers in Balb/c mice.* *(**A**) Histological examination on liver tissue (hematoxylin-eosin staining) 48 h after i.v. administration of indicated amounts of free polymer per 20 g mouse. Development of a fatty liver (black arrows indicate lipid droplets in hepatocytes) after application of 50 µg of non-degradable unPEGylated BM-A1/1 polymer (upper left picture). Its PEGylated variant (PEG-BM-A1/1, upper right picture) in contrast was well tolerated, no inclusion of lipid droplets in liver tissue can be observed. Degradable MK-A1/1 and PEG-MK-A1/1 polymers (lower left and right pictures, respectively) induced no or only marginal histopathological changes in the liver, even at dosages of 100 µg. (**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.*

considerable toxicity remains due to the non-degradability of the polymer. Degradable MK-A1/1, in contrast, is known as highly compatible polymer, tolerated even at the 100 µg dosage by all three mice treated (**Figure 28A**, lower left picture C); only in one of them the onset of a fatty liver was detectable. For the PEGylated variant PEG-MK-A1/1 (**Figure 28A**, lower right picture D) the results were very similar; again one mouse out of three developed a fatty liver and two retained 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.

Additionally, the levels of the liver enzymes alkaline phosphatase (AP) and aspartate aminotransaminase (AST) were determined in the plasma of the mice. Elevated values of these enzymes indicate liver damage. In case of AP no significant differences could be detected in animals treated with MK-A1/1, PEG-MK-A1/1, BM-A1/1 or PEG-BM-A1/1 (dosage: 50 µg/20 g mouse). In contrast, the AST level (**Figure 28B**) of BM-A1/1-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-BM-A1/1, MK-A1/1 or PEG-MK-A1/1 did not differ significantly from those of untreated control mice. This confirms again the advantage of polymer degradability and PEGylation with regard to an improved biocompatibility.

4 Discussion

Recent strategies to improve drug and gene delivery systems focus on the design of more dynamic “stimuli-responsive” formulations (*1, 3*). Similar as viruses in their infection process, these formulations may undergo programmed structural changes (*45, 56, 91*), which are induced by the particular micro-environment such as an intracellular reductive environment (*92-94*), enzymes (*95*), altered pH (*53, 56, 96*), or are introduced externally by physical means (eg. temperature, light or magnetic field). The acidification of endosomes has been capitalized to trigger hydrolysis of acid-labile bonds in drug carriers and conjugates, such as ortho esters (*53, 97, 98*), acetals (*55-57*) or hydrazones (*50, 96, 99*). Their high adaptiveness make this kind of systems so successful.

4.1 Development of a versatile pH-sensitive PEGylation reagent and its application on polyplexes

Among other applications, the strategy of the stimuli responsive system has been used for pH-determined intracellular deshielding of non-viral gene vectors (*100*). Surface shielding of gene vectors by PEG (*81*) or other hydrophilic polymers (*101*) has been found to be very helpful for systemic circulation and reduced toxicity, but hampers intracellular delivery. Therefore removal of the shield after delivery of the gene vector to the target cell would be desirable.

4.1.1 Synthesis and characterization of PEG-A-MAL as a novel acid-sensitive PEGylation reagent

In this work the synthesis of a new acetal linked PEGylation reagent is described, that undergoes acidic triggered cleavage and is shown to be advantageous for reversible shielding of gene delivery particles. The novel PEGylation reagent **9** was synthesized following the steps shown in **Scheme 1**, after synthetic procedures were established using 2-methoxyethanol instead of mPEG. The Buchwald Hartwig cross coupling reaction is a powerful method for substitution of aromatic bromines with piperazine (*102*). In this report the Buchwald Hartwig amination reaction for

substitution of the *p*-bromobenzaldehyde mPEG acetal with the monoprotected piperazine proved to be an efficient procedure affording the desired product with an 82 % yield. To our knowledge it is the first Buchwald Hartwig cross coupling reaction with a polymeric compound.

The pH existing in endosomes varies with the type of endosomal vesicle and the applied formulation (*84, 84, 85*). Therefore hydrolysis studies with PEG-A-MAL were performed at several pHs to determine changes in the hydrolysis rate in dependence of the pH. As shown in **Figure 4A** PEG-A-MAL hydrolysis underlied a strong pH specificity with a half-life of approximately 1 h at pH 7.4 and less than 3 min at pH 5. At pH 5.5 and 6, approximately the pHs existing in PEI containing endosomes (*84, 85*), PEG-A-MAL degraded with a half-life of less than 5 and 10 min, respectively (**Figure 4A**). **Figure 4B** shows the hydrolysis rates of PEG-A-MAL at defined time points as a function of the pH. For pH 5 at all three time points – 5, 10 and 15 min – the reagent showed almost complete degradation, while for pH 7.0 between 96 and 80 % remained intact during the indicated time periods. These data clearly demonstrate the pH dependence of the degradation rate of PEG-A-MAL.

4.1.2 Acid-labile PEGylation of polyplexes

Since these hydrolytic studies showed the PEG-A-MAL linker to be suitable for acid-specific hydrolysis, the reagent was used for PEGylation of different mercapto-modified polycations, used as transfection reagents. Thereby, the reagent turned out to be compatible with all polycations tested, including PEIs. This highlights the versatility of the reagent, and points out the benefit over similar reagents which could not be coupled to PEI (*50*). Kinetic hydrolysis studies performed with the PEG-A-PEI25K conjugate showed similar pH specificity (at 37 °C, half-life of about 2 h at pH 7.4, and 3 min at pH 5.5) as found for PEG-A-MAL. Thus this conjugate provides sufficient storage stability on the one hand and allows polyplex deshielding even in PEI-buffered endosomes of pH 5.5 to 6 on the other hand. This observation was made both for branched PEI25k (this work) and linear PEI22K conjugates (data not shown) and contrasts to previous work with acid-labile hydrazone linkers, where PEI triggered pronounced hydrolysis of the linker also at neutral pH (*50*).

From previous work (*50, 81*) it is known that for the shielding of polyplexes it is often not necessary to substitute all the polycation by its PEGylated version. As the

amount of the PEG conjugate needed depends on the amount of PEG on the polycation as well as the PEG chain length and sort of polycation, we investigated empirically which would be the optimal ratio for the different PEG-A-polycation conjugates. In titration experiments with varying amounts of PEG-A-polycation in the polyplexes the optimal amount of shielding conjugate in the formulation was determined. Ideal ratios ranged between 20 and 30 %, depending on the conjugate used. For polyplexes composed of unmodified PEI22K and PEG-A-PEI25K for example, 20 % of the PEG-conjugate containing 1.5 acetals, i.e. 3 PEG chains per PEI, were found to be most convenient. With this amount of PEG polyplexes showed proper and persistent shielding at physiological pH but at the same time were deshielded quickly at the acidic pH of 5. The acid-labile PEG-A-PEI25K conjugate, which was chosen for further experiments, was then incorporated into DNA polyplexes additionally containing the targeting conjugates EGF-PEI or Tf-PEI to investigate if the acid-labile PEG is also compatible with EGF- and Tf-ligands. As the polyplexes retained their pH-specific deshielding characteristics also in presence of the targeting conjugates, they were finally tested for receptor mediated uptake into cells. EGF containing polyplexes and Tf containing polyplexes have been previously shown to be efficiently internalized into EGFR-overexpressing Renca cells (*83, 103*) and transferrin receptor overexpressing K562 cells (*74, 81*), respectively. To obtain sufficient surface neutralization the pH-sensitive (PEG-A-PEI25K) or non-reversible (PEG-S-PEI25K) shielding conjugate was included into the polyplex at 20 % (w/w; PEI); resulting in well-shielded particles with a zeta potential of +3 mV and particle sizes in the range of 200 nm. In order to follow the deshielding process, these particles were incubated at pH 7.4, representing the physiological pH of the blood, or at pH 5, representing endosomal pH. At pH 7.4 all polyplexes maintained their PEG shield for the 2 h time period, a time spread after which receptor mediated uptake should be completed (*104*). At pH 7.4 additional analysis of the polyplexes after 4 h was performed (data not shown) to assure stability of the PEG shield during the incubation time in cell culture experiments. Polyplexes did not deshield within this time. Also the polyplexes containing the non-reversible shielding conjugate PEG-S-PEI25K did not change their biophysical properties at pH 5 (data not shown). In contrast, the particles containing PEG-A-PEI25K at pH 5 within 30 min formed aggregates and increased in zeta potential, indicating deshielding. The reversibly shielded (PEG-A-PEI25K) polyplexes were found to have approximately 10-fold

enhanced gene transfer efficiency compared to stably shielded polyplexes when tested on the two different cell lines, Renca-EGFR cells and K562 cells. This enhancement is not due to extracellular polyplex deshielding, as transfection medium was replaced by fresh culture medium within a time period where PEG shield is still intact, but due to intracellular PEG removal. Further evidence therefore gave transfection results of experiments where medium was changes already after two hours. Here the same trends in transfection efficiency were obtained (data not shown). This is consistent also with previous results using pH-labile hydrazone linkages (*50*). The novel PEG-A-MAL reagent **9** described in this study, however, presents a more versatile tool for pH-sensitive PEGylation.

4.1.3 Outlook

Beyond the polycations investigated in this study other mercapto-modified compounds may be modified for the development of more dynamic, pH-responsive drug and gene delivery systems. PEGylation is, in any case, a technique becoming more and more popular not only regarding gene therapy, but also in other areas of medicine. Especially in case of the emerging group of biotech protein drugs as for example cytokines (interferons, interleukins, grows factors, erythropoietin), hormones (insulin) or antibodies (*105*) (**Table 3**), PEGylation is a preferential technique. It affords enhanced stability (physically, chemically, proteolytically) and solubility, improved bioavailability (concerning absorption, renal filtration, immunological clearance) and reduced toxicity (immunogenicity, antigenicity).

name	*company*	*drug*	*indication*
Neulasta ®	Amgen	PEG 20 kDa + granulocyte colonystimulating factor	chemotherapy associated neutropenia
Pegasys ®	Roche	PEG 40 kDa + interferon α-2a	hepatitis-C
PegIntron ®	Schering Plough	PEG 12 kDa + interferon α-2b	hepatitis-C
Somavert ®	Pfizer	PEG + human growth hormone receptor antagonist	acromegaly
Adagen ®	Enzon	PEG 5 kDa + adenosine deaminase	severe combined immunodeficiency disease
Oncaspar ®	medac	PEG + L-asparaginase	acute lymphatic leukemia
Cimzia ®	UCB Pharma	PEG + antiTNF-α humanized antibody fragment	rheumatoid arthritis and Crohn's disease
Mircera ®	Roche	PEG + erythropoietin beta	symptomatic anemia in adult patients with chronic kidney disease

Table 3. ***Examples for PEGylated protein drugs on the market (106).***

However, proteins as for example enzymes are biologically active substances, whose activity depends on the accessibility of an active site in the molecule. Consequently, covalent attachment of large molecules like PEG might hamper biological activity. In this case, reversible PEGylation with versatile reagents like PEG-A-MAL might be of interest. These reagents are applicable not only for endosomal targeting, but also other sites of decreased pH can be addressed like tumors (*107*), infarct sites (*108*) or inflamed tissue (*109*). Acid reversible PEGylation thus demonstrates an exiting tool for various fields of application.

4.2 Degradable gene vectors for improved biocompatibility

Polycations used as non-viral vectors often suffer from low gene transfer efficiency or – if they are efficient – possess considerable toxicity. Thus, it is a major challenge in non-viral gene therapy to combine both desired qualities in one vector – the required efficiency of high molecular weight polycations like PEI22K, and the good biocompatibility of low molecular weight oligocations (*33*). The aim of this work was to design degradable polycations of appreciable size, which are composed of small oligomeric units connected via reversible acetal linkages. While maintaining their high molecular weight in the extracellular space, thus providing efficient protection and tight packaging of the nucleic acid and mediating its uptake into the cell, intracellularly these vectors should degrade into small biocompatible decomposition products. Different approaches (i.e. vectors decomposing with different velocities and upon different stimuli) have already been investigated, but currently the optimal specific timing (minutes, hours or days) and location of such a degradation (vesicles, cytoplasm, nucleus) remain unclear (*54, 84*). For example, vector decomposition depending on endosomal milieu, has to proceed fast enough to ensure that polymer degradation is complete before endosomal release into the pH-neutral cytoplasm takes place. This means, vector decomposition has to occur within a quite short time scale (minutes to less than an hour), as an efficient endosomal release of polyplexes must take place before the endosome matures into an endolysosome destroying the delivered nucleic acid. Too rapid degradation of the polymeric carrier which protects the nucleic acid and facilitates its release from the endosome would also be counterproductive. In essence, concerted polymer degradation and endosomal release is hypothesized to be preferred for pH-sensitive carriers. Moreover, it is

difficult to make general statements on optimal location and timing, as the demands depend on the particular objective and target (endosomal DNA delivery / cytoplasmic RNA delivery) and also on the cell type. It also remains to be determined in which specific case which molecular trigger for degradation (reductive cleavage of disulfide bonds (*54, 92*), cleavage upon acidification (*67-69, 110*), hydrolysis of ester bonds (*33, 60, 63, 111*)) is most suitable, alone or in combination.

Disulfide containing vectors for example, which respond with degradation to reductive environment, are widely used. These systems claim to be stable when circulating in the oxidizing extracellular space but disintegrate when taken up into reducing intracellular compartments as the cytosol, the nucleus and in endo-/lysosomes. However, cleavage of the disulfide bond also can occur already before the vehicle is taken up into the cell. It is known that disulfide reduction of macromolecules yet begins at the cell surface, due to cell surface-associated redox enzymes expressed at the plasma membrane (*54, 92*). Reduction at the outside of the cell would cause a loss of DNA due to inadequate cellular uptake and lead to degradation of the insufficiently protected DNA. Saito et al. (*92*) also reported from a group that observed rapid cleavage of disulfides already in the blood (*112*). The disulfide bond can be made more stable by introducing sterically challenging shielding moieties in order to prevent the preterm reduction. However, this is a delicate operation, as the disulfide bonds might than easily become too stable for the intracellular cleavage resulting in an inefficient decondensation of DNA complexes in the endosome and a decreased endosomal escape (*92*). These facts might be especially problematic as the intracellular level of the reducing agent glutathione strongly depends on the tissue type and therefore varies considerably between different cell lines (*113*).

Another kind of biodegradable polymer is based on esters as cleavable linker moieties. These vector systems undergo unspecific hydrolysis in aqueous environment or are degraded by esterases. Degradation of esters takes longer than that of disulfides, as esters possess half-lives in the range of days (*60*); therefore these vectors have positive influence rather on the long term toxicity than on the acute one. Additionally, degradation of ester based polymers is also not fast enough to facilitate the release of DNA from the complexes inside the cell which might hamper the nuclear import. Finally, ester hydrolysis can occur extracellularly as well

as intracellularly and therefore shows no specificity to any special targeted compartment.

A class of gene vectors designed to degrade rapidly upon a specific stimulus in the endosome but to remain stable during the circulation in the blood, are the pH-sensitive vectors. However, these vectors are still rather rare. A main reason therefore surely is the lack of suitable linkages, that show appropriate rapid hydrolsis induced by only a slight decrease in pH as it is existent between cytosol and endosomes (*1*). In the current work we focused on this acidification of endosomal compartments as a specific trigger for polymer degradation.

4.2.1 Design, synthesis and characterization of acid-degradable acetal based polycations for use as gene vectors

For this purpose two different acetal based linkers (BAA and MK) were chosen, by which OEI800 units were oligomerized in order to obtain larger acid-hydrolyzable polymers. The acetone ketal containing linker MK (*70*) has previously been introduced as reversible protein cross-linking agent, the *p*-methoxy-benzaldehyde acetal diacrylate ester BAA (*71*) has been applied before in acid-cleavable polymer particles. In case of BAA, the obtained OEI-BAA polymers contain both the acid-labile acetal groups and hydrolyzable ester groups. If polyplexes might not be degraded completely until they are released from the endosome, the remaining polymer can be hydrolyzed in the cytosol by ester hydrolysis also at neutral pH.

Studies on the acetal cleavage confirmed that the acetal containing polymers MK-OEI and BAA-OEI degrade in a pH-dependent manner, with very fast degradation kinetics in the acidic environment of pH 5 (approximately 3 minute half-life at body temperature) and much slower at physiological pH of 7.4 (3.5 hours half-life for OEI-BAA and 5 hours for OEI-MK). In contrast, the acid-stable control polymers OEI-BM (for OEI-MK) and LT-OEI-HD (for OEI-BAA) degraded under the same acidic conditions either very slowly (LT-OEI-HD) or not at all (OEI-BM).

With regard to their application as gene vectors, DNA binding and compacting capacity of the polymers was determined by ethidium bromide (EtBr) exclusion assay and by particle size and zeta potential analysis. At c/p ratios >1 stable, positively charged particles (i.e. sizes of 100-300 nm, positive zeta potential of 23 – 30 mV)) were obtained in salt-free buffer. The EtBr exclusion assay correlated well

with the size measurements: for polymers that showed weaker DNA-binding affinity (e.g. OEI-BAA polymers) in the EtBr assay, slightly larger particles were detected by size measurements than for those made from polymers with high DNA-binding ability (e.g. MK-A1/1). The firmer the DNA was condensed by the polycation, the smaller the polyplexes were in size. To further elucidate if there is a difference between polymers with high and low DNA-binding affinity, the influence of ionic strength on the polyplexes was investigated. Sizes of polyplexes from polymers with strong DNA binding (MK-A1/1, BM-A1/1) were not influenced by the addition of salt, while those prepared from weakly DNA-binding polymers (BAA polymers, at low c/p also LT-OEI-HD) started to grow in size and aggregated, as it is also known for standard PEIs (*114, 115*). Overall, the polyplexes showed biophysical characteristics in the range of standard PEI22K particles and thus should be suitable for use as gene vectors.

Polyplex transfection efficiency and toxicity were tested using B16F10 and Neuro2A cells. Concerning the compatibility of the formulations, the acid-degradable polymers showed a clear advantage over acid-stable analogs: the acetal containing polymers (OEI-MK and OEI-BAA) were significantly less toxic than their stable controls. This effect became more apparent with increasing c/p ratios. Regarding gene transfer efficiency, we found that polymerization of the very poorly transfecting OEI800 to larger polycations boosts transfection efficiency levels dramatically, both for acid-stable linkages (OEI-BM, LT-OEI-HD) as well as for the acid-degradable (OEI-MK, OEI-BAA) ones. Due to better biocompatibility, for acid-labile polymers the c/p ratios leading to maximum transfection efficiency were slightly higher than for the stable counterparts.

The OEI-BAA polymer series, containing both the acid-labile methoxy-benzaldehyde acetal and hydrolyzable *ß*-aminopropionic ester bond, displayed the most encouraging potential as transfection agent. For example, polymer BAA-2/1-3d mediated gene transfer more effectively than the control ester polymer LT-OEI-HD (with similar m.w.) at all c/p ratios tested. The smaller polymer BAA-1/1-3d which showed lower transfection efficiency at low c/p, with increasing c/p ratio exceeded not only BAA-2/1-3d, but also the golden standard linear PEI22K; however, the stability of BAA-1/1-3d polyplexes against aggregation needs to be improved. In contrast, the BAA-1/1-1.5d variant provides stable polyplexes. While these are less effective than BAA-2/1-3d polyplexes at low concentration, they reach comparable values at higher c/p ratios, without mediating any toxicity. This makes especially the

BAA-1/1-1.5d polymer interesting concerning the promising efficiency/toxicity balance.

In Neuro2A cells, a general low transfection efficiency of medium-sized polyplexes (formed in HBG) is observed, presumably resulting from a general insufficient polyplex cellular uptake. This can be overcome by salt-induced aggregated polyplexes of PEI22K and OEI-BAA polymers. Once more, BAA polymers provided highest gene expression levels, at lowest cytotoxicity.

Additionally, for BAA-2/1-3d as a representative for the BAA line, and for MK-A1/1 the influence of the proton pump inhibitor bafilomycin A1 on transfection efficiency was investigated. Reporter gene expression of BAA-2/1-3d polyplexes in B16F10 cells was diminished significantly by the inhibition of endosomal acidification. The same was also observable for PEI22K, where the lack of proton influx into the endosome prevents performance of the proton sponge effect of PEI (*87*). The fact that bafilomycin A1 has the same impact on transfection efficiency of PEI22K as of BAA-2/1-3d polyplexes suggests, that also for BAA polymers high transfection levels rely at least partially on their ability to capture protons. In contrast, bafilomycin caused no significant alterations on gene expression of MK-A1/1 polyplexes, implying that in this case proton sponge activity does not play a decisive role for transfection efficiency.

In vivo toxicity studies with the free polymers confirmed the encouraging biocompatibility of the acetal containing polymers, found the *in vitro* experiments. Dosages (100 µg/20 g mouse) which were lethal in case of BM-A1/1, PEI25K or PEI22K and caused severe damage in liver tissue in case of LT-OEI-HD, were well tolerated in case of MK-A1/1 and BAA-2/1-3d polymers. This emphasizes the advantage of acid-degradable polymers over the controls also *in vivo* and makes them interesting candidates for use as safe gene transfer agents *in vivo*. Effectiveness in the *in vivo* situation however remains to be evaluated.

Taken together, these data clearly show an advantage of degradable acetal-linked polymers compared to acid-stable ones. The chosen acetal linkage exhibits favorable hydrolysis kinetics: the synthesized polymers provide adequate stability for DNA polyplex formation resulting in efficient gene transfer *in vitro*, but also sensitivity towards a slight decrease in pH in endosomes, which causes polymer degradation and thus improves biocompatibility. Potential bottlenecks beyond biodegradability, like particle aggregation in salt, insufficient endosomal uptake of polyplexes or

deficient endosomal release are challenges that have to be managed by a sophisticated design of the polyplexes. Insufficient endocytosis of polyplexes might be overcome by the integration of suitable ligands to boost specific polyplex uptake into the target cells. Deficient endosomal release, which is still a major limit even for PEI-type polymers despite their proton sponge activity (*85*), might be overcome by incorporation of endosomally active components (*116-118*). Inadvertent particle aggregation can be prevented by pH-responsive PEGylation (*50, 110, 117*). Such strategies have to be investigated in the context of the polymeric carriers presented here, to yield polyplexes with suitable characteristics for *in vivo* gene transfer.

4.2.2 Optimization of the physicochemical properties of MK-1/1 for improved transfection properties

The MK-A1/1 polymer seemed to provide an excellent starting point for further optimization attempts. This polycation shows the high biocompatibility of degradable polymers outclassing common PEI polymers in this regard, but its efficiency is rather low compared to the golden standard PEI22K. To improve transfection properties, the following different aspects were considered to deserve closer attention and were therefore analyzed separately:

4.2.2.1 Increased size and amine density

A direct correlation between molecular weight and transfection efficiency has been reported previously by different groups (*33, 59*) and seemed to be interesting also for MK-A1/1. While MK-A1/1 by far exceeds OEI800 concerning polymer size, it is still inferior to standard PEI22 or PEI25, due to considerable polymer fractions in the low m.w. range. Consequently, decreasing the ratio of these low m.w. fractions in favour of the high m.w. fractions might improve the polymer`s characteristics. Indeed, independent of the method of increasing the average m.w. (fractionation of MK-A1/1 or direct synthesis of polymers with larger sizes like MK-A1.2/1, MK-A1.5/1), this procedure led to remarkable improvements in transfection efficiency especially at low c/p ratios. For the application of higher amounts of cross-linker in the polymer syntheses (MK-A1.2/1, MK-A1.5/1) there is set an upper limit. Former research has already demonstrated that, depending on the linker and the monomer units chosen, there exists a particular limit for the linker/monomer ratio upon which gelation occurs (*60*). Actually, the fractionation method is especially interesting because not only the

polymer size but also its polydispersity is influenced favorably, thus making the polymer molecularly better defined (*119*). Despite the positive influence on transfection efficiency by the increased m.w., the high biocompatibility of the MK-A polymer is not affected negatively at all.

Another aspect to mention in this context is the proton sponge activity, found for example for the highly efficient PEI polymers (*49, 58*). This attribute facilitates endsosomal release and is associated with a high density of protonable amines. The amine density of a polymer can be influenced by the size of the monomeric units it is composed of: when MK-A1/1 is synthesized, in the polymerization process primary amines of OEI800 react via Michael addition with the maleimido functions of the cross-linker. This results in a loss of protonable OEI800-amines in the polymer, which is unfavorable regarding DNA compacting ability (*60, 120*) and endosomal escape mechanisms (*49*). Additionally, the presence of quite spacious linker molecules further decreases the amine density in a polymer compared to an analog linker-free polymer. In case of MK-A1/1 this can be in part antagonized by replacing the OEI800 by larger PEI1.8K units. That way, the ratio of protonable amines/linker molecules is shifted towards the amines, i.e. to an increased density of amines. This facilitates the efficient compaction of DNA into small, stable polyplexes essential for the protection of DNA, efficient cellular uptake and – in conclusion – for efficient gene transfer. It has to be taken into account that the enhancement of amine density is a tightrope walk, as very high charge densities, found for example in PEI22K, PEI25K or PEI750K, destroy cellular membranes and therefore make polymers toxic (*31*). Indeed, also for BM-B1/1 and MK-B1/1 it is not only the transfection efficiency that rises, but also toxicity shows a slight increase, compared to the corresponding OEI800 based polymers (BM-A1/1 and MK-A1/1). It remains to be investigated whether this lower polymer compatibility can be overcome for example by PEGylating the polymers. This seems to be probable, as PEGylation was also applied successfully in case of BM-A1/1 and MK-A1/1.

4.2.2.2 PEGylation

PEGylation is a common method to reduce toxic effects of polyplexes (*38*), but has the drawback of abrogating transfection efficiency at the same time (*39, 100*). As

mentioned above, this handicap can be circumvented by reversibly linking the PEG onto the polycation. PEG, which prevents polyplex toxicity and provides particle stabilization during the circulation, finally is cleaved upon a defined stimulus and does not hamper subsequent steps of the transfection process. It has been demonstrated that transfection efficiency of PEI polyplexes could be restored by acid cleavable PEG-shieldings in the past (*50, 110*). Mostly, such a reversible PEGylation requires supplementary synthesis steps or special, sophisticated PEGylation reagents. In our case, the introduction of a pH-responsive PEG-shield into the MK-A1/1 polymer was a simple procedure as it could be performed concomitantly to the synthesis of the polymer itself, thus avoiding additional purification and production steps. In contrast to the control polymer PEG-BM-A1/1, where the stable PEGylation lowered polymer toxicity and also transfection efficiency, the acid-labile PEGylation in MK-A1/1 surprisingly not only reduced toxicity, but also improved transfection efficiency compared to the non-PEGylated MK-A1/1. Further, PEG-MK-A1/1 was well tolerated also *in vivo*, when tested for biocompatibility in Balb-c mice. It represents therefore an interesting candidate for future transfection experiment *in vivo*.

4.2.2.3 Hydrophobization

According to viruses, which possess special membrane disruptive domains to facilitate their endosomal release, MK polymers were also equipped with such a feature. It is known from literature that hydrocarbon chains, applied in this context, can boost transfection efficiency of polycations (*121, 122*). Therefore different amounts of membrane active, hydrophobic hexyl acrylate moieties were reacted onto MK-A1/1 or PEG-MK-A1/1. While low amounts of HA (HA/OEI ratio of 2.5/1) had no appreciable influence on *in vitro* transfection efficiency or toxicity, high amounts (10/1 ratio) caused extreme toxicity. Interestingly, it was observed, that PEGylated HA variants tended to exhibited slightly higher toxicity than PEG-free analogs. This might be explained by a pronounced tenside-like effect of polymers that combine hydrophilic (PEG) and hydrophobic (HA) moieties. With their highly surface-active characteristics these polymers might cause substantial damage in cell membranes, subsequently leading to cell death. Even though some of these polymers showed surprisingly high transfection levels, exceeding the golden standard PEI22K, they are – in this form – not convenient for use as gene vectors due to their high toxicity.

Here, further efforts are necessary to restore the high biocompatibility of the original MK-A1/1.

Taken together, we applied different methods to improve transfection efficiency of the highly compatible acid-degradable gene vector MK-A1/1. Increasing m.w. or amine density of MK-A1/1, as well as the attachment of acid-cleavable PEG, turned out to be encouraging methods to boost reporter gene expression *in vitro*. Especially the pH-sensitive PEGylation turned out to be very promising and is worth to be examined in *in vivo* transfection experiments.

5 Summary

Non-viral gene delivery systems have emerged to promising tools in gene therapy. Despite many advantages of non-viral vectors over viral ones, it still rests a major challenge to boost transfection efficiency to the levels obtained by virus mediated gene transfer. Hence it appears obvious to adopt the characteristics of non-viral vectors to those of viruses. This implies the incorporation of bioresponsive elements, which confer to the system a higher flexibility and permit to cope with the different – often even oppositional – requirements the vector has to meet before reaching its target. The aim of this thesis was accordingly to equip “artificial viruses” with pH-sensitivity. By the application of acetal chemistry this concept was realized, regarding two different aspects: firstly the acid-reversible PEGylation of polyplexes, and secondly the acid-triggered degradation of polymeric gene carriers.

PEGylation prevents polyplexes from undesired interactions, both with physiological components in the patient and among themselves. On the other hand stable PEG shielding hampers endosomal release of endocytosed complexes and diminishes transfection efficiency. In contrast, the PEG-A-MAL reagent developed in this work, allows to PEGylate polycations reversibly, providing stable polyplex shielding in the extracellular compartments but, after cleavage upon acidification in the endosome, facilitating endosomal release. The reagent was compatible with different kinds of polycations commonly used as gene vectors (e.g. PEI, PLL) and also tolerated the concomitance of targeting ligands. In cell culture experiments on two different cell lines transfection efficiency was enhanced 5- to 14-fold with PEG-A-MAL shielded polyplexes compared to stably shielded analogs, highlighting the potential of this versatile reagent.

In terms of improved vector biocompatibility degradable systems have recently attracted more and more interest. Herein, vectors composed of oligoethylenimine units cross-linked via acetal containing linkers are described. The acid cleavable acetal functions provide appropriate hydrolysis kinetics for polymer degradation upon a slight drop of pH, as it occurs in endosomes. Two polymer lines have been established: the MK-line based on an acetone ketal and the BAA-line based on a *p*-methoxy-benzaldehyde acetal. Both variants showed significantly reduced toxicity compared to stable analogues in cell culture experiments. Transfection efficiency of the novel polymers was promising, especially in case of some BAA polymers which

were able to compete with the golden standard PEI22K. In case of MK polymers, alterations in the physicochemical properties could further enhance transfection efficiency. The most promising approach represented the acid-sensitive PEGylation of MK-A1/1, which could be achieved conveniently in a one-step synthesis. The resulting polymer showed not only increased gene transfer efficiency but also reduced toxicity.

Selected polymers were finally tested for biocompatibility *in vivo*. Liver tissue analysis of mice treated with the plain polymers confirmed the improved tolerance of acid degradable polymers over acid-stable analogs.

6 Appendix

6.1 Abbreviations

AP	alkaline phosphatase
AST	aspartate aminotransaminase
BAA	1,1-bis-(2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane) linker
BM	1,8-bis-maleimidodiethyleneglycol linker
CMV	cytomegalovirus
c/p ratio	cation/plasmid (weight/weight) ratio
Da	dalton
DCM	dichloromethane
DMEM	Dulbecco´s Modified Eagle´s Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EGF	epidermal growth factor
EGF-PEI	epidermal growth factor covalently linked to PEI25K via a heterobifunctional 3.4 kDa PEG spacer; EGF/PEI ratio: 1.2/1
EGFR	epidermal growth factor receptor
EtBr	ethidium bromide
EtOAc	ethyl acetate
FACS	fluorescence activated cell sorting
FCS	fetal bovine serum
GLDH	glutamate dehydogenase
GPC	gel permeation chromatography
HA	hexyl acrylate

HBG	Hepes-buffered glucose (5 % (w/v) glucose, 20 mM Hepes, pH 7.4)
0.5 HBS	Hepes-buffered glucose and Hepes-buffered saline 1/1 (v/v)
HBS	Hepes-buffered saline (5 % glucose (w/v), 150 mM NaCl, 20 mM Hepes, pH 7.4);
HD	1,6-hexanediol diacrylate linker
Hepes	*N*-(2-hydroxyethyl)piperazine-*N*`-(2-ethane sulfonic acid)
HPLC	high pressure liquid chromatography
HT-OEI-HD1	OEI-HD polymer synthesized at 60 °C (= high temperature) and at molar input ratio of OEI/HD = 1/1
IR	infrared spectroscopy
kDa	kilodalton
LT-OEI-HD	OEI-HD polymer synthesized at 22 °C (= low temperature)
ME-A-MAL	*N*-{3-[4-(4-{1-[bis-(2-methoxyethoxy)]methyl}phenyl)pi-perazinyl]-3- oxopropyl} maleimide
MFI	mean fluorescence intensity
MK	2,2-bis(*N*-maleimidoethyloxy) propane linker
Mn	m.w. by number
Mp	m.w. at the peak maximum
mPEG	poly(ethylene glycol) monomethyl ether, average m.w. of 5 kDa
MTT	methylthiazoletetrazolium salt
m.w.	molecular weight
Mw	m.w. by weight
N/P ratio	molar ratios of PEI nitrogen to DNA phosphate
OEI800	oligoethylenimine with an average m. w. of 800 Da
OEI-HD	HD-linked OEI800
PBS	phosphate-buffered saline

pCMVLuc	plasmid encoding luciferase under control of the CMV promoter/ enhancer
PDI	polydispersity index
pDNA	plasmid DNA
PEG	poly(ethylene glycol)
PEG5K	poly(ethylene glycol), average m.w. of 5 kDa
PEG-A-MAL	*N*-(3-{4-[4-(1-{bis[monomethoxy poly(ethylenoxy)]}methyl) phenyl]piperazinyl-]3-oxopropyl)maleimide
PEG-A-PEI25K	mPEG 5 kDa acetal conjugate with PEI25K
PEG-MAL	maleimide modified mPEG: methoxy poly(ethylene glycol) maleimido average m.w. of 5 kDa
PEG-SH	thiol modified mPEG: methoxy poly(ethylene glycol) thiol, average m.w. of 5 kDa
PEG-S-PEI25K	mPEG of 5 kDa covalently and stably attached to PEI25K
PEI	polyethylenimine
PEI22K	linear PEI with an average m. w. of 22 kDa
PEI1.8K/2K/10K/25K/50K	branched PEI with an average m.w. of 2/10/25/50 kDa
PEI-SH	thiolated PEI
PLL	poly-(L-lysine), hydrobromide,
PLL58K	PLL, average m.w. of 58 kDa
RES	reticulo endothelial system
RLU	relative light units
RNA	riboxy nucleic acid
r.t.	room temperature
s.d.	standard deviation
SEC	size exclusion chromatography
SPDP	succinimidyl 3-(2-pyridyldithio)propionate
THF	tetrahydrofuran
TEA	triethylamine
Tf	transferrin

Tf-PEI	transferrin covalently linked to PEI25K via a heterobi-functional 3.4 kDa PEG spacer; Tf/PEI ratio: 0.9/1
TNBS	2,4,6-trinitrobenzenesulfonic acid
v	volume
Vp	elution volume at peak maximum
w	weight
λem	emission wavelength
λex	exitation wavelength

6.2 Publications

6.2.1 Original papers

Knorr, V.; Allmendinger, L.; Walker, G.F.; Paintner, F.F. and Wagner, E. (2007)
An acetal based PEGylation reagent for pH-sensitive shielding of DNA polyplexes
Bioconjugate Chem. 18 (4) 1218-1225

Knorr, V.; Russ, V.; Allmendinger, L.; Ogris M. and Wagner, E. (2008)
Acetal linked polyethylenimines for use as pH-sensitive gene carriers.
Bioconjugate Chem., in press

Manuscript in preparation

Knorr, V.; Ogris M. and Wagner, E.
An Acid Sensitive Ketal-based Polyethylene Glycol-Oligoethylenimine Copolymer Mediates Improved Transfection Efficiency at Reduced Toxicity.

6.2.2 Poster presentation

Knorr, V.; Allmendinger, L.; Walker, G.F.; Paintner, F.F. and Wagner, E. (2007)
pH-sensitive shielding of DNA polyplexes using a novel acetal-based PEGylation reagent. Poster 141, XVth Annual Congress of the European Society of Gene and Cell Therapy (ESGCT), October 27 – 30, 2007; Rotterdam.

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9 Curriculum Vitae

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