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ORIGINAL ARTICLE

Novel degradable oligoethylenimine acrylate ester-based pseudodendrimers for in vitro *and* in vivo *gene transfer*

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A novel class of cationic hyperbranched polymers, containing branched oligoethylenimine (OEI 800 Da) as core, diacrylate esters as linkers and oligoamines as surface modification, was synthesized and evaluated regarding their structure–activity relationship as gene carriers. We show that pseudodendritic core characteristics as well as different surface modifications on the core influence DNA-binding ability, cytotoxicity and transfection efficiency. As most promising gene carrier, the pseudodendrimer HD O, that is, the OEI 800 Da core modified with hexane-1,6-diol diacrylate and surface-modified with OEI 800 Da, was identified. HD O exhibits efficient DNA-condensing ability to nanosized polyplexes (100–200 nm), low cytotoxicity, a degradation half-life of 3 days at 37 °C at physiological pH and in vitro *reporter gene-expression levels similar to high molecular weight linear and branched polyethylenimines (PEIs) (LPEI and BPEI).* In vivo *studies in mice reveal that HD O/DNA polyplexes upon i.v. tail-vein injection have the potential for transfection of tumor tissue at levels comparable to that obtained with LPEI. Importantly, HD O was better tolerated than LPEI, while transgene expression was more tumor-specific and much lower in all other investigated organs, especially in the lung (15 000-fold lower compared with LPEI).*
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Introduction

Gene therapy offers a promising tool for treating various forms of cancer and a number of inherent diseases, although the clinical use is still mainly restricted due to the lack of safe and efficient gene delivery systems.[1] Nonviral gene carriers might be advantageous due to pharmaceutical issues, such as low cost of synthesis and easy scale-up, but they show poor transfection efficiency levels in comparison to their viral counterparts.[2,3] Thus, an excellent *in vivo* transfection activity at low toxicity should be the major considerations regarding the design of novel nonviral gene delivery devices. Recently, many degradable polymers have been generated,[4–14] aiming to reduce the toxicity profile while maintaining transfection efficiency levels comparable to nondegradable gene vectors like polyamidoamine dendrimers[15–17] or optimized polyethylenimines (PEIs).[18,19] For example, promising results have been obtained[6,9] by coupling low molecular weight oligoethylenimine 800 Da (OEI) with short diacrylate linkages, thus combining the beneficial low cytotoxic properties of OEI with the higher transfection efficiency of higher molecular weight PEIs. The hydrolysis of the ester bonds may occur under physiological conditions, and the low cytotoxicity was correlated to the degradation behavior of these polymers.[6,9] A technical weakness of this polymer approach is the polydispersity, that is, the heterogeneity in molecular weight, which is inherent to the applied procedure in polymer synthesis. The design of dendrimers is an elegant solution to obtain well-defined polymers with narrow molecular weight distribution. As an example, polyamidoamine dendrimers have already successfully been applied for gene delivery.[16] In the current paper, we report the design of a novel family of hyperbranched polymers, namely pseudodendrimers, which consists of OEI in the center functionalized with an excess of degradable dioldiacrylates to form a pseudodendritic core that is subsequently modified on its surface with different oligoamines. Although these conjugates do not have the typical symmetric dendritic structure based on AB_2 monomers, they follow the dendritic concept based on a branched structure, assembled by adding a generation of monomer to a core unit.[20] As gene delivery vectors, these pseudodendrimers may have several advantages: (i) amines, presented in the periphery of the pseudodendrimers can interact well with DNA and form nanoscaled pseudodendrimer/DNA complexes; (ii) the high density of secondary and tertiary amines in the focal point may facilitate endosomal escape of pseudodendrimer/DNA complexes ('proton sponge' effect);[21]

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(iii) the dendritic branches containing ester bonds allow degradation of pseudodendrimers under physiological conditions, increasing their biocompatibility with regard to *in vivo* application; (iv) pseudodendrimers can be easily modified further to improve transfection efficiency, for example, using targeting ligands or shielding domains.

The pseudodendrimer family reported here was first studied regarding its structure–activity relationship. We show that pseudodendritic core characteristics as well as different surface modifications among the pseudodendritic core influence both transfection efficiency and cytotoxicity *in vitro*. Most importantly, we identify in our screening, a pseudodendritic structure that exhibits high *in vitro* transfection efficiency at low cytotoxicity and very encouraging *in vivo* gene expression levels after systemic application in s.c. tumor-bearing mice.

Results

Synthesis and characterization of pseudodendrimers

Pseudodendrimers were synthesized in two steps (Figure 1). In the first step, pseudodendritic cores were generated by Michael addition of oligoethylenimine 800 Da (OEI) with a 20-fold molar excess of dioldiacrylate. Due to the excess of dioldiacrylate, cross-linking between OEIs was prevented, and a branched structure with reactive end groups was created. The different diacrylates of ethyleneglycol, butane-1,4-diol and hexane-1,6-diol applied as shown in Figure 1a display increasing hydrophobicity (ED < BD < HD). In the following step, each OEI core (ED, BD and HD core) was further modified on its surface by adding an excess of either ethanolamine (E), spermidine (Sp), spermine (S) or OEI (O) as shown in Figure 1b. For further studies within the HD core conjugates, ethylenediamine (EDA), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), oligoethylenimine 1200 Da (O1200) and oligoethylenimine 1800 Da (O1800) were also used. The different amines were selected to stepwise increase the number of nitrogen per surface modification unit. Conditions for synthesis including reaction temperature, reaction time as well as concentrations of educts were optimized in preliminary studies (data not shown). Under the chosen reaction conditions, neither gelation nor precipitation occurred. After purification by dialysis and lyophilization, all products were well water-soluble. The nomenclature of pseudodendrimers is derived from the core moiety followed by the surface amine used.

The structural composition of the resulting products was analyzed using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The calculated molar diacrylate/OEI and surface amine/OEI ratios, the percentage of esters and yields of pseudodendrimers after synthesis are listed in Supplementary Table S1 (Supplementary information). In general, approximately 10 diacrylates were coupled per OEI (diacrylate/OEI ratio), resulting in a tree-like pseudodendritic core structure. The ratio of surface amine to OEI was lower compared to the ratio of diacrylate to OEI for all conjugates, indicating that more than one pseudodendritic branch reacts per surface modification unit (surface amine/OEI ratio). In fact, it seems that per surface amine, two to three amine functionalities have reacted per pseudodendritic core. Interestingly, ethanolamine that contains only one amine function per molecule also has a lower surface amine/OEI ratio, indicating that intramolecular side reactions of the dioldiacrylate during pseudodendritic core synthesis cannot be prevented. The esters, which present the degradable bonds of the polymers, might also aminolyze or hydrolyze during synthesis and purification. Therefore, the ester content of all conjugates was analyzed; it was ranging around 90% of the theoretical value except for ED O, which contained only 71% of ester bonds after synthesis. As expected, for all three different cores with an increasing number of nitrogen per surface amine, a slightly decreasing percentage of ester bonds was found (HD core: 97% (E) to 87% (O), BD core: 94% (E) to 85% (O) and ED core: 90% (E) to 71% (O)), which can be explained by aminolysis during pseudodendrimer synthesis catalyzed by an increasing amount of unprotonated amino groups.

The molecular weights (gel permeation chromatography) of the pseudodendrimers are listed in Supplementary Table S2 (Supplementary information). In general, all pseudodendrimers had much lower molecular weights than the standard PEIs, LPEI and BPEI. With an increasing number of nitrogen per surface modification unit, all pseudodendrimers showed increasing molecular weights. For example, within HD core polymers, molecular weights of 1410 Da for HD Sp, 2613 Da for HD S and 4138 Da for HD O were found. However, the shorter the hydrocarbon spacer in the coupled dioldiacrylate was, the higher were the resulting molecular weights.

For conjugate HD O degradation, kinetic studies were carried out at physiological pH of 7.4 at 37 °C over a time period of 7 days. The extent of degradation was determined using ^{1}H NMR spectroscopy. After 3 days, approximately 50% of the ester bonds were degraded, and after 7 days, almost no residual ester bonds were present, demonstrating that HD O is degradable under *in vitro* conditions (Supplementary Figure S1, Supplementary information).

Biophysical characterization of pseudodendrimers

For efficient gene delivery, the gene carrier must be able to condense DNA to form complexes suitable for cell entry. This formation was studied in an ethidium bromide exclusion assay measuring reduction of relative fluorescence as a function of the conjugate/plasmid (c/p) w/w ratio (Supplementary Figure S2; Supplementary information). When investigating the influence of the surface modification on DNA-binding ability (Supplementary Figure S2A), a similar DNA-binding pattern was found for ED, BD or HD cores: surface modification with ethanolamine (E) containing one nitrogen showed the lowest DNA-binding ability followed by spermidine (Sp) and spermine (S) modification, with three and four nitrogen per coupled oligoamine, respectively. OEI (O) surface modification (18 nitrogens per coupled oligocation) resulted in the best DNA-compacting properties, indicating that an improved complex formation capability was found with an increasing number of nitrogen per coupled oligocation (Supplementary Figure S2A; Supplementary information). This corresponded to the finding that an increasing number of nitrogen per coupled oligoamine resulted in stepwise increased ζ-potentials resulting in an enhanced DNA-condensing

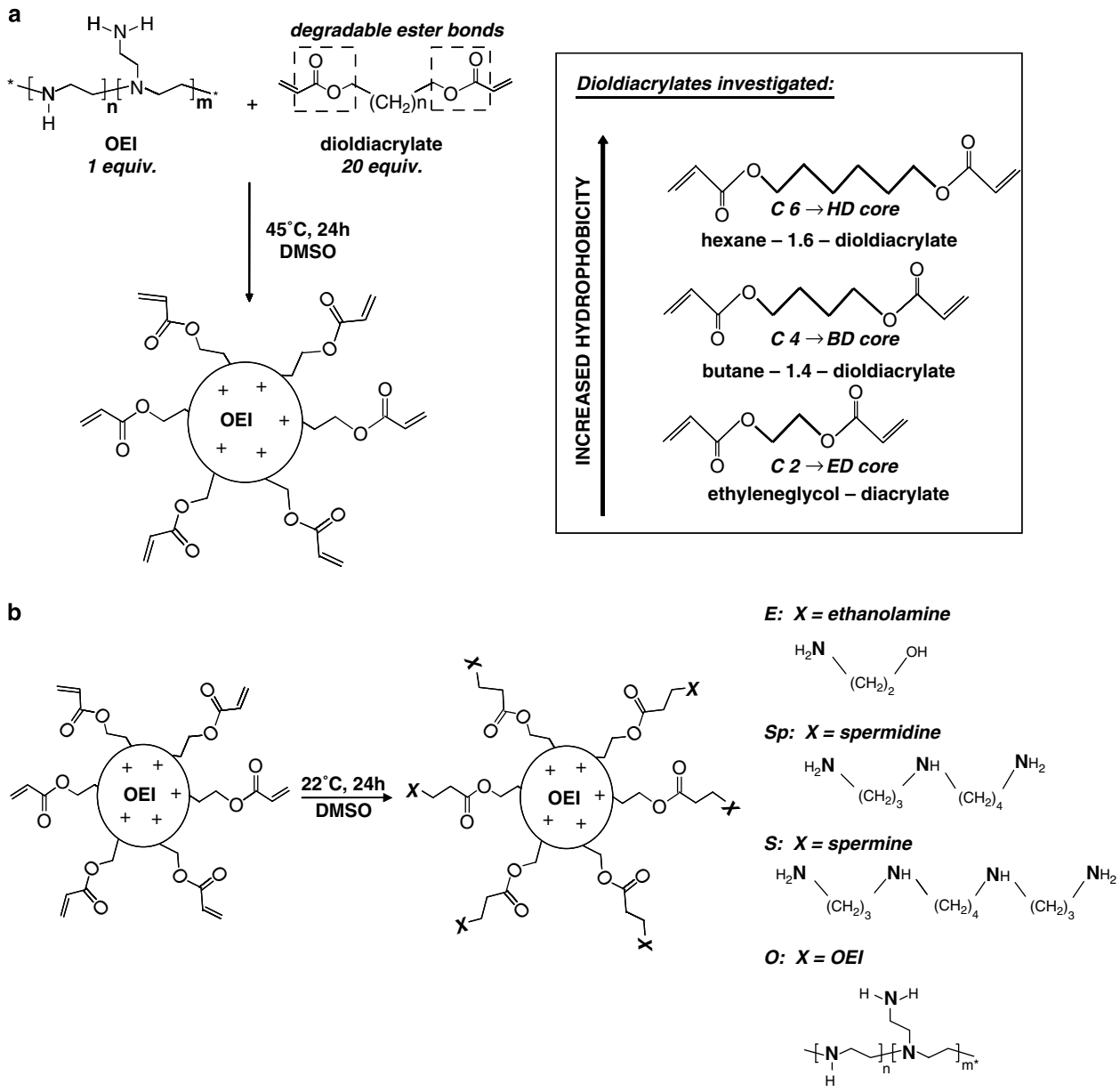


Figure 1 Pseudodendrimer concept. (**a**) Pseudodendritic core synthesis using oligoethylenimine 800 Da (OEI) and an excess of different dioldiacrylates. (**b**) Pseudodendritic surface modification with different oligoamines as surface modification units.

ability (see below and Supplementary Table S3; Supplementary information).

Regarding the influence of the core characteristics on DNA binding, it was found that a larger hydrocarbon spacer in the coupled dioldiacrylate increased DNA-compacting capabilities (Supplementary Figure S2B; Supplementary information). Thus, HD core conjugates exhibited the best DNA-compacting abilities followed by BD and ED core conjugates, resulting in stepwise decreased capacity of complex formation. Notably, this effect was representative for ethanolamine (E), spermidine (Sp) and spermine (S) surface modifications, whereas OEI (O) as coupled oligocation showed similar good DNA-compacting abilities upon all pseudodendritic cores, indicating that the influence of the surface modification unit was able to mask core characteristics.

Mean diameters (DLS) and ζ-potentials of DNA polyplexes with conjugates at a c/p ratio of 2 are listed in Supplementary Table S3 (Supplementary information). HD core conjugates exhibited polyplex sizes around 160 nm except for the ethanolamine-modified HD core (531 nm). Although we cannot exclude loose DNA compaction, the tendency of HD E to aggregate at low c/p ratio is consistent with the fact that BD E results in small polyplex sizes, although it shows a lower DNA-binding ability. BD core conjugates showed polyplex sizes around 200 nm, while ED core conjugates resulted in decreasing polyplex sizes with an increasing number of nitrogen per surface modification unit (ED E: 894 nm; ED O: 181 nm), correlating with their increasing DNA-condensing ability. At c/p ratio of 2, all conjugates based on the three different pseudodendritic cores exhibited

stepwise increasing ς-potentials with an increasing number of nitrogen per coupled oligocation as shown in Supplementary Table S3 (Supplementary information). Hence, measured ς-potentials of HD core conjugates were ranging from +2.7 mV (HD E) to +29 mV (HD O), of BD core conjugates from +6.8 mV (BD E) to +25.1 mV (BD O) and of ED core conjugates from −8.8 mV (ED E) to +24.0 mV (ED O).

Biological characterization of pseudodendrimers in vitro

To investigate the influences of core characteristics and the surface modification on cytotoxicity, a lactate dehydrogenase (LDH) assay and an ATP assay were performed. This allows parallel monitoring of cell membrane damage (LDH assay) vs metabolic activity of cells (ATP assay) after treatment with various concentrations of pseudodendrimer.

Figure 2a shows the influence of the hydrocarbon spacer in the coupled dioldiacrylate, demonstrating that pseudodendritic core hydrophobicity correlates with cytotoxicity. In the particular example shown in the figure, ethanolamine was applied as representative surface amine. While BD and ED cores did not show any LDH release from the cytoplasm of treated cells, the HD core bearing a C6 spacer exhibited membrane damage in a dose-dependent manner (Figure 2a, left). In parallel, metabolic activity of treated cells decreased with the length of the C-spacer, shown by the rapid decrease of cell viability after HD E treatment (Figure 2a, right). BD E and ED E exhibited rather low or no decrease of metabolic activity in cells after conjugate treatment even at the highest concentration of 1 mg ml^{-1}.

Investigating the influence of the surface modification on cytotoxicity, it was found that with an increasing number of nitrogen per coupled oligoamine, a stepwise increased cytotoxic effect was detectable. This can be seen best in the ED core series (Figure 2b). The ethanolamine conjugate bearing one nitrogen per surface modification unit (E = 1 N) showed neither a decrease of metabolic activity nor an increase of LDH release; whereas, the spermidine surface modification (Sp = 3 N) resulted from a concentration of 0.5 mg ml^{-1} upwards in decreased cell viability, while no membrane damage was detectable. A higher number of nitrogen upon the

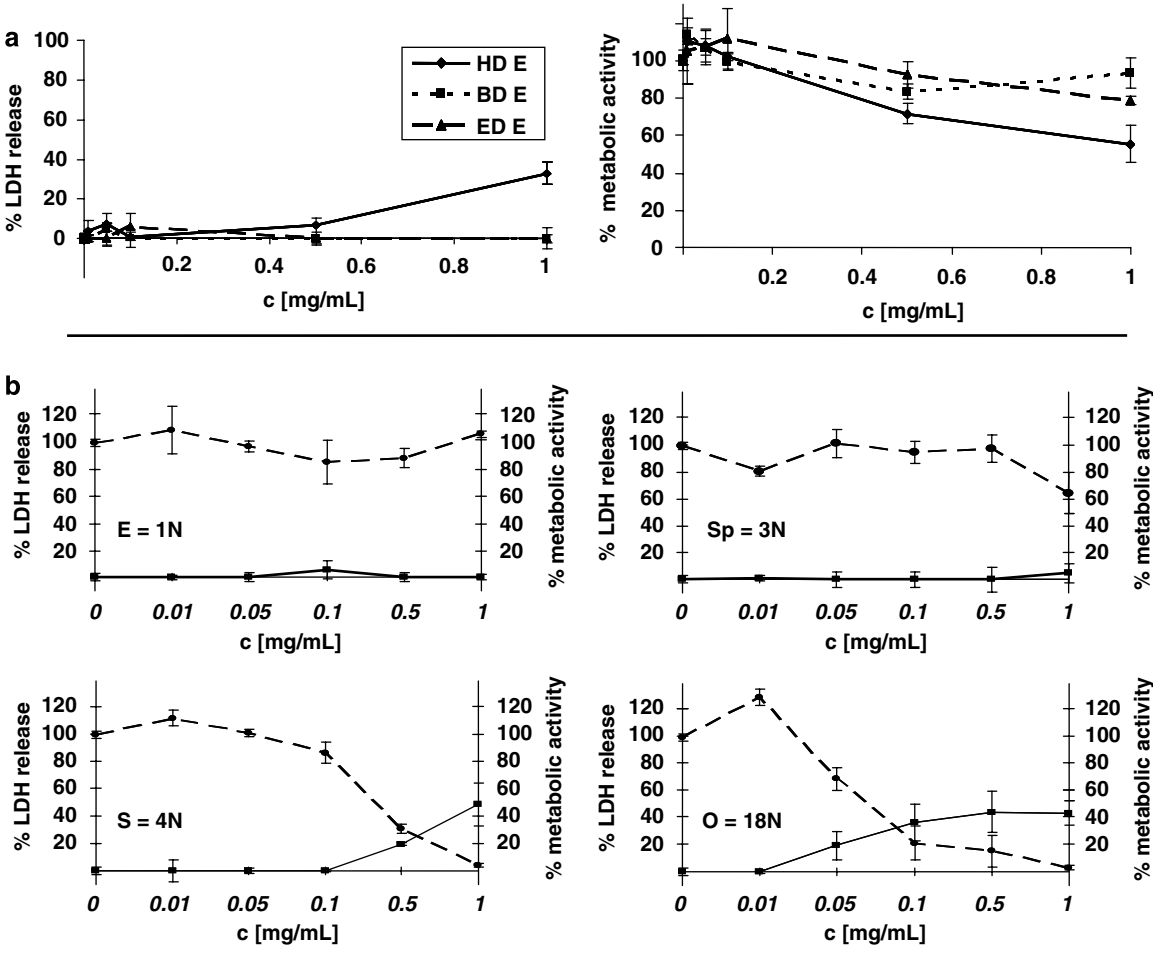


Figure 2 Structural influences of plain pseudodendrimers on cytotoxicity. The lactate dehydrogenase (LDH)-release assay monitors cell membrane damage and the ATP assay the metabolic activity of cells after treatment with pseudodendrimers. All experiments were performed in Neuro2a cells in triplicates. (**a**) Pseudodendritic core: cytotoxicity increases with an increasing aliphatic spacer in the coupled dioldiacrylate, i.e., increasing core hydrophobicity enhances toxic effects. (**b**) Pseudodendritic surface modification: cytotoxicity increases with an increasing number of nitrogen per coupled oligoamine. LDH release, solid line; ATP assay, dashed line.

surface obviously boosted cytotoxic effects consisting of membrane damage and decreased metabolic activity of cells after pseudodendrimer treatment as shown by spermine (S = 4 N) and OEI (O = 18 N) surface modifications.

Transfection efficiency and parallel cytotoxicity studies of all pseudodendrimers were carried out in B16F10 and Neuro2a cells. The plasmid pCMVLuc encoding for firefly luciferase was used as reporter gene and cells were incubated with polyplexes in the presence of 10% fetal calf serum for 4 h. For LPEI and BPEI, taken as references, we found in both cell lines an optimized c/p (conjugate/plasmid (w/w)) ratio of 0.8 (that is, nitrogen/phosphate (N/P) ratio of 6) (Supplementary Figure S3; Supplementary information).

Figure 3 shows in Neuro2a and B16F10 cells, both the influence of pseudodendritic core characteristics and the influence of the surface modification unit on transfection efficiency and cytotoxicity at a c/p ratio of 2. This c/p ratio represents optimized conditions for most conjugates (Supplementary Figures S4–S6; Supplementary information). For all surface modifications, we found that HD core polymers showed significantly enhanced transfection efficiency over BD and ED core counterparts, whereas improved transfection levels from ED to BD core conjugates were not significant. The only exception found was for ED O that showed highest transfection levels in B16F10 cells, which was a cell type-specific effect (Figure 3b, left). According to the influences of the surface modification on transfection efficiency, we found within the HD and the ED core conjugates, a stepwise rising transfection level with an increasing number of nitrogen per coupled oligocation (E < Sp < S < O). This effect was almost independent of investigated c/p ratios (Supplementary Figures S4 and S6; Supplementary information). BD core conjugates, however, did not show such a direct correlation and mediated in both cell lines moderate transfection. Furthermore, we did not find within BD and ED core conjugates significant cytotoxic differences for all surface modifications (Figure 3, right). Nevertheless, increasing core hydrophobicity decreased metabolic activity, that is, increased cytotoxicity. Thus, after HD E, HD Sp and HD S polyplex treatment, metabolic activity of cells significantly decreased as compared to their ED and BD core counterparts. Interestingly however, the best transfection conjugate HD O complexed with DNA did not exhibit a decreased metabolic activity, and cell viability was maintained high. To investigate this effect more in detail, we studied additional five surface modification units with the HD core (Figure 4). Apparently, high transfection levels at low cytotoxicity depend on an optimized balance of core characteristics and surface amine. This is a fact in the case of HD O, exhibiting highest transfection levels at

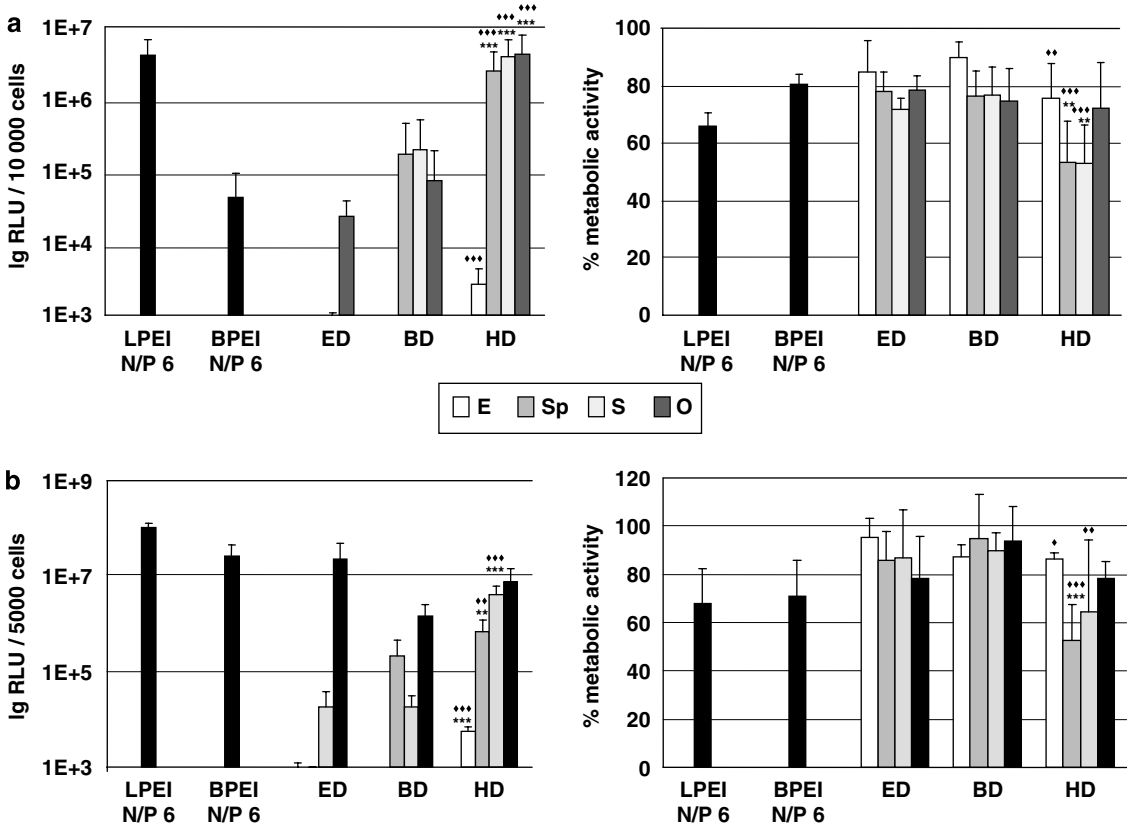


Figure 3 Influence of the hydrocarbon spacer in dioldiacrylates and the influence of the surface modification unit in pseudodendrimers on transfection efficiency (left, Luciferase assay) and metabolic activity (right, methylthiazoletetrazolium (MTT) assay) in Neuro2A cells (**a**) and B16F10 cells (**b**) in the presence of 10% serum, 24 h after transfection. All polyplexes were prepared at c/p—(conjugate/plasmid, w/w) ratio of 2 in HBG. Linear and branched polyethylenimines (LPEI and BPEI) were applied at optimized N/P ratio of 6, which represents a c/p ratio of 0.8. Data expressed as mean values (±s.d.) out of three independent experiments each performed in triplicates. RLU/number of cells represents the measured relative light units referred to the number of cells at the time of cell plating (analysis of variance (ANOVA), Duncan's test, $^{**}P<0.05$, $^{***}P<0.001$ ED vs HD, $^{\blacklozenge\blacklozenge}P<0.05$, $^{\blacklozenge\blacklozenge\blacklozenge}P<0.001$ BD vs HD).

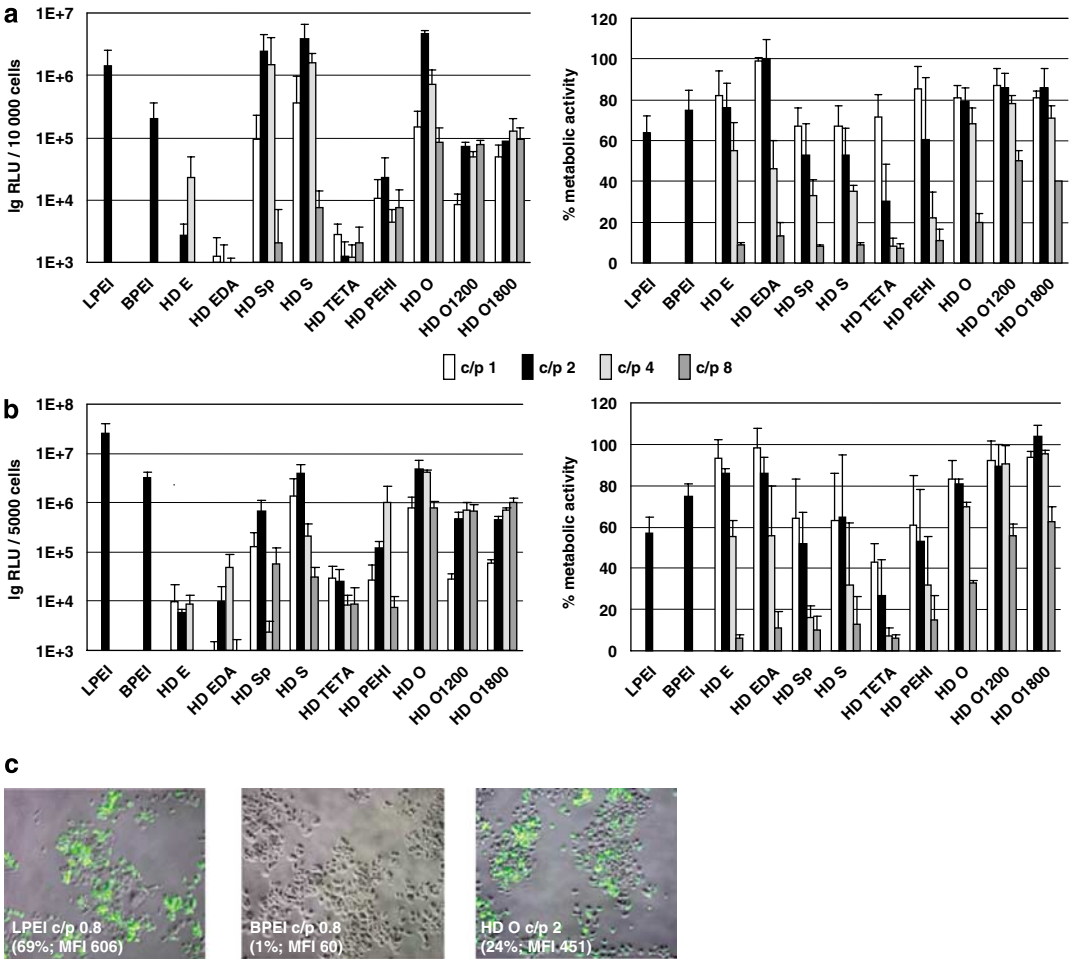
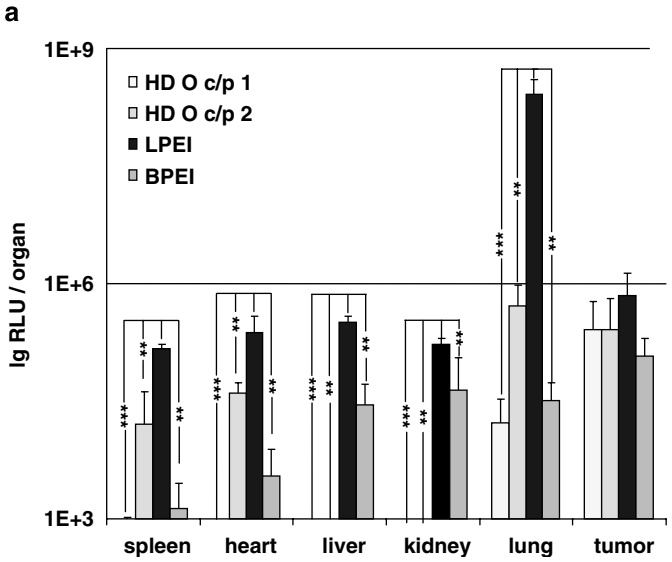


Figure 4 *In vitro* transfection efficiency (left, Luciferase assay) and metabolic activity (right, methylthiazoletetrazolium (MTT) assay) of HD core pseudodendrimers, linear polyethylenimine (LPEI; 22 kDa) and branched polyethylenimine (BPEI; 25 kDa) in Neuro2a cells (**a**) and in B16F10 cells (**b**) in the presence of 10% serum 24 h after transfection. Polyplexes were formed at indicated c/p (conjugate/plasmid, w/w) ratios in HBG; LPEI and BPEI were applied at optimized N/P ratio of 6, which represents a c/p ratio of 0.8. Data were expressed as mean values (±s.d.) out of three independent measurements each performed in triplicates. RLU/number of cells represents the measured relative light units referred to the number of cells at the time of cell plating. Surface modifications upon the HD core: ethanolamine (E), ethylenediamine (EDA), spermidine (Sp), spermine (S), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), OEI 800 Da (O), OEI 1200 Da (O1200), OEI 1800 Da (O1800). (**c**) Enhanced green fluorescent protein (EGFP) expression studies of LPEI, BPEI and HD O in Neuro2a cells at their optimized c/p ratios (in HBS). MFI represents mean fluorescence intensity of cells at 24 h after transfection measured by fluorescence-activated cell sorter analysis. The percentage of EGFP-positive cells is indicated in parentheses.

low toxicity in both Neuro2a and B16F10 cells. Importantly, HD O showed in both cell lines similar high reporter gene expression levels as the standard PEIs. Especially in Neuro2a cells, HD O exhibits similar high transfection levels as LPEI and was about 100-fold more efficient than BPEI (all at their optimized c/p ratios). Polymers may differently affect the protein levels of transfected cells due to differential toxicity and cell proliferation. Therefore, in separate experiments analogous to Figures 3 and 4, transfection levels were quantified side by side as relative light units (RLUs) per mg of protein and RLU per number of seeded cells (Supplementary Figures S7 and S8; Supplementary information). The gene expression pattern of the different polymers in the two normalizations was almost identical; thus, ruling out significant effects of protein levels. Figure 4c shows the comparison of these polyplexes at enhanced green fluorescent protein (EGFP) expression levels in Neuro2a cells. Mean fluorescence intensities mediated by HD O and LPEI polyplexes were high, whereas BPEI, in contrast, showed a much lower fluorescence signal. LPEI and HD O also showed high percentage of EGFP-positive cells (69 and 24%, respectively, as determined by flow cytometry), while BPEI polyplexes only transfected 1% of cells.

Gene expression in vivo

Due to their high transfection levels *in vitro*, we investigated HD S and HD O also *in vivo*. First studies on acute toxicity in mice revealed that HD S as plain conjugate was highly toxic after i.v. application, resulting in lethality of mice, whereas HD O was well tolerated. Same results were found in preliminary studies using polyplexes. Therefore, HD O was further investigated for its *in vivo* gene transfer in tumor-bearing mice. We conducted our experiments by i.v. injection of complexes of HD O (c/p ratios of 1 and 2) as well as LPEI and BPEI as references (c/p ratio of 0.8 = N/P 6) using pCMVLuc



b

	(AST + ALT)/GLDH	AP
HBG	4.9 (± 1.4)	225 (± 37)
HD O c/p 1	6.3 (±2.6)	190 (±47)
LPEI c/p 0.8	11.7 (±4.7)	189 (± 15)
BPEI c/p 0.8	17.3 (±8.6) *	253 (±9)

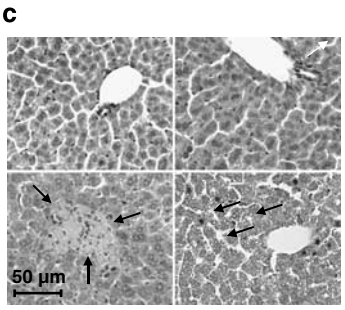


Figure 5 (**a**) Luciferase gene expression 24 h after i.v. administration of HD O at c/p ratios of 1 and 2, linear and branched polyethylenimines (LPEI and BPEI) at c/p ratio of 0.8 (N/P 6) into mice-bearing Neuro2A s.c. tumors. Luciferase gene expression is presented as relative light units per organ or tumor (RLU/organ) (mean ± s.d., $n = 3$ animals per group). Tumor weights ranged between 250 and 300 mg. Two nanogram of recombinant luciferase correspond to 10^7 light units. ***$P < 0.001$ and **$P < 0.01$ for LPEI vs HD O and BPEI (analysis of variance (ANOVA), Duncan's test). (**b**) Liver enzyme levels in serum 24 h after polyplex treatment. Alkaline (ALT) and aspartate (AST) aminotransaminases, glutamate dehydrogenase (GLDH) and alkaline phosphatase (AP). Data represent mean values (+s.d., $n = 3$ animals per group), except buffer ($n = 4$)) (**c**) Histological examinations (hematoxylin–eosin staining) on liver tissue 24 h after polyplex treatment (each as a representative per group). HD O c/p ratio of 1 shows no changes in liver histology as compared to a buffer-treated control. Necrotic areas in the liver are indicated by the arrows after LPEI c/p ratio of 0.8 treatment. Lipid droplets in hepatocytes in the case of BPEI-treated mice with c/p ratio of 0.8 show a microvesicular fatty liver (arrows) (upper left, control (HBG); upper right, HD O c/p ratio of 1; lower left, LPEI c/p ratio of 0.8; lower right, BPEI c/p ratio of 0.8).

as reporter gene. Complexes were prepared at a final DNA concentration of 200 µg ml^{-1} in HEPES buffered glucose (HBG), with 50 µg DNA applied per mouse. Mice receiving polyplexes showed no signs of acute toxicity. A 6% loss of body weight was observed 24 h after injection of HD O polyplexes at c/p ratio of 1, whereas at c/p ratio of 2, we found 14% decreased body weight, showing in both cases, no significant differences compared to LPEI (11%) and BPEI (14%). Figure 5 presents luciferase reporter gene expression in main organs and tumor tissue 24 h after i.v. application of polyplexes. Consistent with previous work, the application of LPEI polyplexes led to luciferase gene expression in all investigated organs but was predominantly found in the lung (>100-fold higher compared to all other organs and tumor tissue).[22,23] BPEI exhibited reporter gene expression mainly in tumor tissue, liver and lung at lower levels compared to LPEI. The pseudodendrimer HD O resulted at both c/p ratios in transgene expression that was found in tumor tissue at gene expression levels comparable to LPEI. Importantly, for HD O at c/p ratio of 1, far lower luciferase gene expression was observed in the lung, and no significant transgene expression was observed in any other organ. The reasons for the differences remain to be clarified; reporter gene expression may not be in direct correlation to the body distribution pattern, as previously described.[24,25] In the current case, positive charges of polyplexes cannot be the sole explanation for the observed gene expression patterns since HD O at c/p ratio of 2 still shows a more favorable tumor/lung relation as compared to LPEI.

The biocompatibility of HD O (c/p ratio of 1) was further assayed in tumor-free mice. No increase of liver enzyme levels (AST, ALT, GLDH and AP) was found as compared to a buffer (HBG)-treated control. In contrast, LPEI and BPEI (both c/p ratio of 0.8) showed increased liver enzyme levels (for BPEI being statistically significant, $P < 0.05$, analysis of variance (ANOVA), Duncan's test), indicating beginning necrotic changes (Figure 5b). Pathohistological examinations underline these findings as shown in Figure 5c, where no changes in liver histology were found in the case of HD O as compared to the control. In contrast, LPEI leads to necrotic changes in liver tissue as indicated by the arrows and BPEI results already after 24 h in microvesicular fatty liver shown by inclusion of lipid droplets within the hepatocytes (see arrow).

Discussion

Polymers frequently used for nonviral gene transfer, like PEI[18,19,26] or polyamidoamine dendrimer[15–17] are not degradable and exhibit significant toxic effects after systemic application.[27] Therefore, the aim of this study was to synthesize a novel class of nonviral gene delivery vectors that shows low toxic effects at gene expression levels comparable to commonly used standard BPEI and LPEI. Degradable random polymers based on low molecular weight oligoethylenimine 800 Da (OEI) were previously described by several researchers including our group.[9,13,16,28–30] In the current work, we designed better defined pseudodendritic polymers of low molecular weight starting with OEI as core building block, using different dioldiacrylates, which contain degradable ester bonds to form pseudodendritic branches and

added various oligoamines to modify the pseudodendritic cores.

In our studies, we were able to demonstrate the following structure–activity relationships with regard to pseudodendritic core characteristics and surface modifications. We showed that an increasing number of carbon atoms in the aliphatic spacer of the diacrylates was able to increase DNA condensation ability, revealing that an increasing hydrophobicity of the pseudodendritic core plays an important role in DNA binding. This is consistent with previous work by Zhong *et al*.,[7] who found that among their linear poly(ester amine)s investigated, the most hydrophobic ones showed the best DNA-binding ability. Furthermore, an increasing number of nitrogen per coupled oligoamine on the pseudodendritic surface resulted in stepwise rising ς-potentials, which also led to enhanced DNA binding and condensation to nanoscaled polyplexes.

Reduced cytotoxicity is undoubtedly one of the most important issues with regard to *in vivo* application of nonviral gene carriers, but at the same time, high transfection efficiency is also important. We were able to show in our *in vitro* studies that toxic effects and transfection efficiency levels of our pseudodendrimers are based on both pseudodendritic core characteristics and the properties of the surface oligoamine. Decreasing pseudodendritic core hydrophobicity from HD to BD to ED core leads to reduced cytotoxicity, but, in general, also significantly decreased transfection efficiency of these compounds (Figure 3). Taken together, for efficient DNA gene delivery using pseudodendrimers, a hydrophobic core domain is essential that probably facilitates cellular uptake due to hydrophobic interactions with the cell membranes. For endosomal release, other factors besides the buffering capacity of oligoethylenimines ('proton sponge')[21] that include hydrophobic interactions with endosomal membranes[31] or the cationic nature of the polymers[32–34] may play an important role mimicking the membrane-disruptive properties of viruses or natural lytic agents.[35] According to the influences of the surface modification unit on cytotoxicity, we observed that an increasing number of nitrogen per coupled oligoamine led to an increased toxic effect if plain conjugates were used. This might be due to unspecific interactions of pseudodendritic surface amines with cell membranes (Figure 2). In contrast, if complexed with DNA, pseudodendrimers showed a different behavior, since direct interactions were 'hampered' by the condensed DNA, and the characteristics of the resulting polyplexes determined cytotoxicity and transfection efficiency (Figure 3). Thus, stepwise enhanced transfection efficiency with an increase of nitrogen per surface modification unit was observed for ED and HD core conjugates, whereas BD core conjugates showed no direct correlation (also not in a c/p ratio dependent manner). Furthermore, in both cell lines, the different surface amines of HD (but not ED or BD) core conjugates did influence cytotoxicity. These facts indicate that both the pseudodendritic core characteristics together with the surface modification within a polyplex play a determining role for high transfection activity at low cytotoxicity. This is emphasized in the HD core conjugates. For example, in Neuro2a cells cytotoxic effects increased from E to Sp to S and then decreased again for the OEI surface-modified conjugate, meanwhile transfection efficiency levels stepwise increased (Figure 3). Apparently, although a hydrophobic HD core displays toxic effects but is essential for high transfection levels, cytotoxicity of the total polyplexes seem to be 'tuneable' by the surface oligoamine. To study this phenomenon more in detail, we investigated various surface oligoamines upon the HD core (Figure 4). As shown, we found that both cytotoxicity and transfection levels depend on an optimized balance between hydrophobic and hydrophilic domains within the pseudodendrimers: on the one hand, a more hydrophobic structure like HD TETA leads to very high cytotoxicity at low transfection efficiency, similarly HD S exhibits rather high cytotoxicity but also high transfection levels (presumably due to better protonation and DNA binding of S over TETA). On the other hand, only the hydrophilic structure such as the hydrophilic ED core conjugates leads to low cytotoxicity but also moderate transfection levels. However, starting with the hydrophobic HD core, with the increasing number of hydrophilic ethylenimine units in the surface modification (TETA < PEHI < O < O1200 < O1800), the toxicity is reduced. At the same time, gene transfer increases to an optimum level at O (that is, oligoethylenimine 800 Da). Taken together, we consider that for efficient DNA gene delivery using pseudodendrimers, a hydrophobic core domain is needed but has to be in optimal balance with the hydrophilic surface. This design led us to consider HD O as a pseudodendrimer that exhibits efficient DNA-condensing ability to nanosized polyplexes (100–200 nm), low toxicity and reporter gene transfection levels, which are similar to high molecular weight PEIs (LPEI and BPEI). HD O was further investigated according to its degradation behavior *in vitro*, using ^{1}H NMR spectroscopy finding that after about 3 days at physiological pH 7.4 and 37 °C, approximately 50% of HD O degraded in aqueous medium, thereby exhibiting promising degradation properties.

As HD O exhibited low acute toxicity in mice, it was investigated for its *in vivo* gene transfer in tumor-bearing mice. As references, we studied LPEI and BPEI. LPEI is known to reveal highest transgene expression levels in mice with gene expression mainly found in the lung after i.v. application being also responsible for acute toxic effects.[22,27] BPEI was used to analyze the differences in gene-expression levels between branched and linear high molecular weight structures. Transgene expression of BPEI complex was only found in tumor, liver and lung, whereas consistent with previous work, BPEI led to a significant lower gene expression in the lung than LPEI.[36] The pseudodendrimer HD O resulted in reporter gene expression, which was predominantly found in the tumor. At a c/p ratio of 1, much lower luciferase gene expression was found in the lung (20-fold lower than in tumor) and negligible gene expression in all other investigated organs. HD O was well tolerated by mice and pathohistological investigations on liver tissue underline this finding, since no changes in liver histology compared to buffer-treated mice have been found as well as no increase of liver enzyme levels (AST, ALT and AP) were detectable after HD O complex treatment. In contrast, LPEI and BPEI complexes resulted in an increase in liver enzyme levels, indicating beginning necrotic changes in liver tissue already after 24 h consistent with previous literature.[27,37] The reasons for

the better *in vivo* tolerability of HD O polyplexes remain to be investigated in detail; besides, its degradability and good biocompatibility (no erythrocyte aggregation in the presence of serum), obvious structural differences including the lower molecular weight compared to standard LPEI or BPEI might influence the toxicity profile. Within the PEI class of polymers, a lower molecular weight has previously been found to provide better biocompatibility.[38,39]

In conclusion, we demonstrated novel degradable cationic pseudodendrimers useful for gene transfer *in vitro* and *in vivo*. In our studies, we observed a specific structure–activity relationship according to their biophysical and biological properties, including cytotoxicity and gene transfer efficiency. Tuning of the pseudodendritic structure toward HD O resulted in a substantial increase of transfection efficiency at low cytotoxicity. Our first *in vivo* studies reveal that plain HD O/DNA polyplexes (without further functional modifications) have the potential for increased specific transfection of tumor tissue at levels comparable to that of LPEI, but exhibiting significantly reduced transgene expression in the lung (15 000-fold lower than LPEI). Moreover, HD O was well tolerated by mice showing best biocompatibility properties.[40] Taken together, this degradable gene carrier may provide an interesting platform for further functional modifications with targeting ligands or shielding domains, while detailed studies according to its biodistribution pattern and degradation behavior *in vivo* are ongoing.

Materials and methods

Chemicals and reagents

Low molecular weight PEI 800 Da water-free (oligoethylenimine, OEI), 1,6-hexandioldiacrylate (HD), 1,4-butandioldiacrylate (BD), 1,2-ethyleneglycoldiacrylate (ED), branched PEI 25 kDa (BPEI), spermidine (Sp), spermine (S), ethylenediamine (EDA), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), deuterium oxide (D_2O) and dimethyl sulfoxide puriss (DMSO) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanolamine (E) was obtained from Fluka (Buchs, Switzerland). PEI 1200 Da (O1200) and PEI 1800 Da (O1800) were from Polysciences Inc. (Warrington, PA, USA). Linear PEI 22 kDa (LPEI) was synthesized by acid-catalyzed deprotection of poly(2-ethyl-2-oxazoline) (50 kDa, Sigma-Aldrich) in analogous form as described[41] and is also available from Polyplus Transfections (Strasbourg, France). All reagents were used without further purification. LPEI and BPEI were adjusted to pH 7 using 1 N HCl (Sigma-Aldrich) prior to use. Cell culture media, antibiotics and fetal calf serum were purchased from Life Technologies (Karlsruhe, Germany). Plasmid DNA pCMVLuc (Photinus pyralis luciferase under control of the cytomegalovirus (CMV) enhancer/promoter) described in Plank *et al*.[42] was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA) was produced and purified by ELIM Biopharmaceuticals (San Francisco, CA, USA). Water was used as purified, deionized water.

Synthesis of pseudodendrimers

Pseudodendrimers were synthesized in two steps. In the first step, the pseudodendritic core was formed by a Michael addition of OEI to dioldiacrylates. The surfaces of the cores were modified in the second step using different oligoamines.

Pseudodendritic core synthesis. Oligoethylenimine was coupled with a 20-fold molar excess of dioldiacrylate to generate a pseudodendritic core. In a typical experiment, OEI (Mw 800) (80 mg, 0.1 mmol). and 1,6-hexandioldiacrylate HD (452.5 mg, 2 mmol) were separately dissolved in DMSO water-free (1.0 ml) under argon (stock solutions). Oligoethylenimine stock solution (100 µl, 0.01 mmol) was mixed with 800 µl DMSO water-free, and subsequently 100 µl HD stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 r.p.m., Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product.

Surface modification. The cores bearing active acrylate groups on the surface were modified with different oligoamines including ethanolamine (E), spermidine (Sp), spermine (S) and OEI (O). For each oligoamine, a 0.6-M stock solution was prepared in DMSO. Pseudodendritic cores (1 ml) were mixed with 0.3 mmol of each oligoamine stock solution (0.5 ml). Reaction proceeded for 24 h at 22 °C under constant shaking (1000 r.p.m., Eppendorf Thermomixer). The obtained products were diluted 1:5 in water, and the pH was immediately adjusted to pH 7 using 1 N HCl. Purification proceeded via dialysis using a Spectra/Por membrane (MWCO 1000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against 4 l of water at 4 °C for 24 h. After 2 h and 20 h, water was exchanged against 4 l of fresh water. The dialysates were collected and freeze-dried. Yields of obtained conjugates were listed in Supplementary Table S1.

Characterization of pseudodendrimers

Resulting products were characterized by ^{1}H NMR spectroscopy. Spectra in D_2O (10 mg ml^{-1}) were recorded on an Eclipse +500 spectrometer (JOEL, Tokyo, Japan) operating at 500 MHz. Degradation of HD O was investigated in an aqueous solution (1 ml, D_2O) at pH 7.4 at 37 °C. Remaining ester content was expressed as a percentage relative to determined ester content after synthesis ($t = 0$ day). Molecular weights of pseudodendrimers were determined by gel permeation chromatography. Gel permeation chromatography analysis 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 × 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^{-1}. Results were evaluated using PSS WinGPC Unity software. Molecular weights (Supplementary Table S2) were measured relative to Pullulan molecular weight standards that were used for preparing a standard calibration curve.

DNA-binding assay
DNA-binding ability of pseudodendrimers was evaluated in HBG (20 mM HEPES, 5% (v/v) glucose, pH 7.4), using an ethidium bromide exclusion assay as described elsewhere.[9] All experiments were run three times.

Cell culture
Cultured cells were grown at 37 °C in 5% CO_2-humidified atmosphere. B16F10 murine melanoma cells and Neuro2a murine neuroblastoma cells were cultured in DMEM (1 g l^{-1} glucose) supplemented with 10% fetal calf serum (v/v) and 1% (v/v) penicillin/streptomycin.

Lactate dehydrogenase and ATP assay
Neuro2a cells were seeded at a density of 10^4 cells in a 96-well plate (TPP, Trasadingen, Switzerland). After 24 h, medium was replaced by 100 µl of fresh medium containing predetermined pseudodendrimer concentrations of 0.01–1 mg ml^{-1}. Per well, 50-µl samples were collected after an incubation time of 4 h, and LDH concentration was determined using a commercial kit (Cytotox 96; Promega, Madison, WI, USA) following the manufacturer's protocol. The percentage of LDH release was defined by the ratio of determined LDH concentration per well over total LDH content in untreated (intact) cells.

Remaining 50 µl of medium per well was used to analyze the ATP content, utilizing a commercial kit (Cell Titer Glo Luminescent Viability Assay; Promega, USA) for the determination of ATP following the manufacturer's protocol. The relative metabolic activity was defined by the ratio of measured ATP content over the ATP content of untreated cells.

Polyplex formation
Plasmid DNA-encoding firefly luciferase (pCMVLuc) was condensed with pseudodendrimers and LPEI or BPEI as references. Final DNA concentration of pseudodendrimer/DNA complexes for *in vitro* studies was 20 µg ml^{-1}, for *in vivo* studies 200 µg ml^{-1}. In brief, polyplexes were prepared by adding HBG-buffered polymer solutions at varying concentrations to an HBG-buffered plasmid DNA solution. Polyplexes were allowed to incubate for 20 min at room temperature.

Polyplex size and ζ-potential measurement
Particle size of pseudodendrimer/DNA polyplexes (in HBG) were determined by dynamic light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK) at room temperature as described previously.[43] Complexes had a final DNA concentration of 10 µg ml^{-1}. The presented data (Supplementary Table S3; Supplementary information) are means of three measurements, with each measurement averaging the data of 10 sub-runs. For estimation of ζ-potential, polyplexes were diluted in 1 mM NaCl to give a final DNA concentration of 2 µg ml^{-1} and were measured using a Malvern Zetasizer 3000HS. The data (Supplementary Table S3; Supplementary information) represent the means of three measurements, averaging the data of five sub-runs.

In vitro *transfection and metabolic activity*
In vitro transfection experiments were performed with either B16F10 or Neuro2a cells. Two parallel transfection series, one for the determination of reporter gene expression (luciferase) and the other for the evaluation of metabolic activity using an methylthiazoletetrazolium (MTT) assay, were carried out in separate 96-well plates (TPP). All samples were done in triplicates in three independent experiments. LPEI and BPEI polyplexes at their optimized c/p ratios for both cell lines were prepared as references.

In a standard experiment, 24 h prior to transfection, B16F10 cells were seeded at a density of 5×10^3 cells and Neuro2a cells at a density of 1×10^4 cells in 200-µl medium per well. At the time of transfection, cells reached a confluency of 60–80%. Before cells were treated with complexes, medium was exchanged against 100-µl fresh medium. Complexes (10 µl) were added directly to the cells and after 4 h, the medium was exchanged against 100-µl fresh one. Transfection efficiency was evaluated 24 h after treatment by measuring luciferase reporter gene expression using a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany) as previously described.[43] Two nanogram of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^7 light units. Protein concentration was determined using a BCA assay (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.[44]

The percentage of metabolic activity of cells after complex treatment was evaluated 24 h after transfection using the MTT assay as described elsewhere.[9] Optical absorbance was measured at 590 nm (reference 630 nm) using a microplate reader (Spectraflour Plus; Tecan Austria GmbH, Grödig, Austria). Metabolic activity was expressed relative to the MTT value for untreated cells taken as 100% metabolic activity of cells.

Enhanced green fluorescent protein expression studies were carried out in Neuro2a cells in a 24-well plate. At 24 h prior to transfection, 4×10^4 cells were plated in 1 ml of medium reaching at the time of transfection, a confluency of 60–80%. Transfection of HD O, LPEI and BPEI polyplexes at their optimized c/p ratios (in HBS) was performed using 1 µg of pEGFP-N1. Twenty-four hours after transfection, cells were viewed on an Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam camera, using appropriate filters for EGFP excitation and emission. Digital image recording and image analysis were performed with the Axiovision 3.1 (Carl Zeiss) software. For the analysis of EGFP expression, cells were washed with phosphate-buffered saline and harvested by trypsin treatment and analyzed as described elsewhere using a CyanADP flow cytometer (DakoCytomation, Kopenhagen, Denmark).[45] Per sample, 3×10^4 gated events were collected, values shown are the average of four transfected wells.

In vivo *studies*
Animal experiments were performed according to National Regulations and approved by the Local Animal Experiments Ethical Committee. Male and female A/J mice were purchased from Harlan Winkelmann (Harlan, Borchen, Germany). Animals were housed in individually vented cages; food and water were provided

ad libitum. For transfection studies, 6-week-old mice were inoculated subcutaneously in the flank with 1×10^6 Neuro2a cells. Experiments started when tumors reached a diameter of 6–10 mm^2. Gene transfer in tumor-bearing mice was carried out as described.[43] After 24 h, mice were killed and main organs and the tumor were resected. Tumor weights ranged between 250 and 300 mg. Luciferase activity was determined using a luminometer (Lumat LB 9507; Berthold Technologies) to obtain RLU per organ and RLU per tumor. Two nanogram of recombinant luciferase (Promega, Germany) correspond to 10^7 light units.

Biocompatibility studies were carried out in 20-week-old mice without tumor. At 24 h after polyplex treatment, mice were killed and blood was collected by puncture of inferior vena cava using heparinized syringes. For histological analysis, mice were perfusion-fixed with formalin solution (4% paraformaldehyde in phosphate-buffered saline), and livers were embedded in paraffin.

For the determination of liver-specific blood enzymes, samples were allowed to clot at 37 °C for 4 h and then centrifuged at 3000 *g* for 20 min at 4 °C. Supernatants were collected for serum analysis. Alkaline and aspartate aminotransaminases (AST, ALT), alkaline phosphatase (AP) and glutamate dehydrogenase (GLDH) were analyzed using standard kinetic UV tests.

Paraffin-embedded livers were cut into 5-µm thick sections and stained with hematoxylin–eosin for histological investigations. Microscopic pictures were taken with an Axiovert 200 inverted microscope (Carl Zeiss) using a $\times 40$ LC Achroplan objective.

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