Stabilized Nonviral Formulations for the Delivery of MCP-1 Gene into Cells of the Vasculoendothelial System

Martin C. Lenter,[1] Patrick Garidel,[2] Jaroslav Pelisek,[3] Ernst Wagner,[3] and Manfred Ogris[3,4]

Received September 8, 2003; accepted December 28, 2003

Purpose. The purpose of this study was to develop a stabilized nonviral gene transfer system for the efficient delivery and expression of monocyte chemoattractant protein 1 (MCP-1) gene in cells of the vasculoendothelial system.

Methods. Plasmid DNA was condensed with polyethylenimine (PEI), conjugates of PEI with polyethylene glycol (PEG), and PEI conjugates with the membrane-active peptide melittin. Surface charge and particle size of the resulting gene transfer particles were analyzed by laser light scattering. Reporter gene studies and toxicity assays were conducted on smooth muscle cells and endothelial cells of human, porcine, or rat origin.

Results. Nonviral gene carriers containing PEI and PEG were developed that could be produced in batches of several milligrams and conveniently stored as frozen samples. Incorporation of PEG into the transfection complex significantly reduced cellular toxicity. The cryoconserved gene transfer particles mediated high expression of luciferase, enhanced green fluorescent protein (EGFP), or secreted alkaline phosphatase reporter genes. Highest reporter gene expression was achieved with PEI polyplexes containing PEG and melittin. The gene for MCP-1 was efficiently delivered into target cells and resulted in expression of up to 125 ng/ml secreted bioactive MCP-1 protein per 50,000 cells.

Conclusions. Gene carriers based on PEI and PEG display reduced toxicity, can be stored in frozen form without loss of biological activity, and can efficiently transfect cells of the vasculoendothelial system. Such gene carriers hold a potential for use in arterial gene transfer and local secretion of MCP-1 as trigger of therapeutic arteriogenesis in arterial occlusion diseases.

KEY WORDS: arteriogenesis; gene transfer; PEG; polyethylenimine; vascular gene therapy.

[1] Boehringer Ingelheim Pharma GmbH & Co. KG, GFB F&E Germany, Genomics & Proteomics Group, D-88397 Biberach an der Riss, Germany.

[2] Boehringer Ingelheim Pharma GmbH & Co. KG, GFB Biopharmaceuticals, Formulation Development, D-88397 Biberach an der Riss, Germany.

[3] Pharmaceutical Biology-Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität, Butenandtstrasse 5-13, D-81377 München, Germany.

[4] To whom correspondence should be addressed. (e-mail: manfred.ogris@cup.uni-muenchen.de)

ABBREVATIONS: L-PEI, linear polyethylenimine, MW 22 kDa; PEG-PEI, block co-polymer of polyethylene glycol (MW 20 kDa) and L-PEI; Mel-PEI, block co-polymer of melittin peptide and branched PEI (25 kDa); PEC, primary porcine endothelial cells; PSMC, primary porcine smooth muscle cells; HUVECs, primary human umbilical vein endothelial cells.

INTRODUCTION

Monocyte chemoattractant protein 1 (MCP-1) plays a key role in the development of new blood vessels after arterial obstruction (1,2). The chemokine attracts monocytes from the circulation that aid in the remodeling of pre-existing arterioles into mature functional collateral arteries. In this process, termed arteriogenesis, intra-arterial shear stress is the driving force of vascular remodeling that gives rise to larger vessel diameter and improved functionality of collateral arteries. This results in a restoration of blood flow and can protect tissue damage by increasing the blood perfusion of target tissue. In different animal models, including minipigs (3) and rabbits (4), the application of MCP-1 protein resulted in earlier growth of collateral arteries following arterial occlusion. The application of MCP-1 protein was mainly performed via a surgically implanted arterial catheter and continuous infusion of the protein for several days. Because prolonged intra-arterial infusion of MCP-1 in a clinical setting appears undesirable, a therapeutic nonviral gene transfer using complexed plasmid DNA would be a viable approach. For this purpose, arterial application of MCP-1 gene could be performed within minutes via a catheter in a single and short therapeutic session ensuring the continuous and local expression of MCP-1 protein over several days. Thus, MCP-1 gene therapy would be a potential alternative to an intra-arterial infusion of MCP-1 protein.

The aim of this study was to develop a nonviral gene delivery system that (i) efficiently transfects cells of the vasculoendothelial system, (ii) is nontoxic, and (iii) can be stored for extended periods of time. We have chosen polyethylenimine (PEI) for packing plasmid DNA into small particles because PEI is known to be highly efficient for transfection of mammalian cells both *in vitro* and *in vivo* (5,6). This system has the advantage of being nonimmunogenic and can be produced in large quantities. PEI polyplexes, however, can exhibit strong toxicity both on cultured cells (7) and after systemic application (8). In agreement with previous observations (9–11), such toxicity can be strongly reduced by including polyethylene glycol (PEG) into the complex. A very welcome "side effect" of incorporation of PEG is the stabilization of polyplexes (10,11), which enables storage in frozen form. In order to increase further the transfection efficiency by enhancing intracellular endosomal escape, the membrane active peptide melittin was incorporated into the polyplexes (12). Transfection activities were studied in primary endothelial cells and smooth muscle cells as well as the A-10 smooth muscle cell line. Luciferase, enhanced green fluorescent protein (EGFP), and secreted alkaline phosphatase were applied as marker genes and MCP-1 as the therapeutic gene.

MATERIALS AND METHODS

Reagents and Conjugates

Linear PEI (L-PEI, 22 kDa) is commercially available from Euromedex (Souffelweyersheim, France). Melittin-PEI (Mel-PEI, melittin peptide in all-D conformation) was coupled via disulfide bonds to branched PEI 25 kDa analogously as described (12). PEG-L-PEI was synthesized by covalently coupling polyethylene glycol (PEG, 20 kDa) to L-

PEI as described recently (10). For DNA polyplex formation, plasmid pSEAP2-control encoding secreted alkaline phosphatase under the SV40 promoter (BD Biosciences Clontech, Heidelberg, Germany; Cat. No. 6052-1) and plasmids pAH7-Luc encoding the Photinus pyralis luciferase, pAH7-EGFP encoding enhanced green fluorescent protein (EGFP), and pAH7-MCP1 encoding the genomic sequence of human MCP-1 (Acc.-no. M37719.1), all pAH7 plasmids expressing the gene under the CMV promoter/enhancer in the same pcDNA3/pZErO-2 framework (Invitrogen, Breda, The Netherlands), were used.

Measurement of Size and Zeta Potential

A Zetasizer 3000HS (Malvern Instruments, Malvern, UK) was used for measurements. For size determination, complexes were diluted to a final concentration of 10 μg/ml DNA with the appropriate buffer; for zeta-potential measurements, the samples were further diluted with 10 mM NaCl to 2 μg/ml DNA. Ten sub-runs were conducted for each measurement, and results were analyzed using Contin software. Functionality of the Zetasizer was checked regularly by calibrated latex beads with defined sizes (20–800 nm, size deviation ±2.5%, Duke Scientific Corporation, Palo Alto, CA, USA) and the zeta potential transfer standard (Malvern, charge deviation measured ±10%).

Cell Culture

A-10 rat smooth muscle cells (DSMZ, Braunschweig, Germany) were cultivated in DMEM supplemented with 20% FCS; primary porcine endothelial cells (PEC) and primary porcine smooth muscle cells (PSMC) were isolated and propagated as described (13). Primary human umbilical vein endothelial cells (HUVECs) were purchased from BioWhittaker (Verviers, Belgium) and cultivated using the EGM bullet kit.

Cell Association Studies by Flow Cytometry

Plasmid DNA was fluorescently labeled with the intercalating nucleic acid stain YOYO-1 using a molar ratio of 1 dye molecule per 300 base pairs (14). The labeled plasmid was thereafter complexed with the indicated PEI conjugates. A-10 cells were seeded at a density of 10^5 cells in 12-well plates (Nunc, Roskilde, Denmark) 1 day prior to transfection. Cells were transfected with YOYO-1 labeled polyplexes in 600 μl of DMEM/20% FCS. After transfection, the medium was aspirated, the cells washed with 2 ml of PBS and trypsinized at 37°C in 1 ml of Trypsin/EDTA solution (Invitrogen, Karlsruhe, Germany). The cell suspension was transferred to FACS tubes and stored on ice until further analysis. Cells were analyzed by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser emitting light at 488 nm. The filter used for emission was a 530/30 nm bandpass. To discriminate viable cells from dead cells and cell debris, appropriate gates for forward and side scatter were applied. Ten thousand gated events were collected per sample; experiments were performed in duplicates.

Viability and Reporter Gene Assays

Cellular viability was measured with the Promega CellTiter-Glo luminescent cell viability assay (Promega, Munich, Germany) according to the manufacturer's recommendations. Expression of luciferase and EGFP was measured as recently described (12). The assay for secreted alkaline phosphatase (SEAP) was carried out by a modified method according to Gee *et al.* measuring the increase in fluorescence after enzymatic conversion from methyl-umbelliferon-phosphate (4-MUP) to methyl-umbelliferon (15). In brief, cell culture supernatant was centrifuged to remove cell debris and heated to 65°C for 10 min to reduce endogenous phosphatase activity. Twenty-five microliters of each sample were placed into a separate well of a black 96-well plate, 25 μl of sample buffer (100 mM glycine, pH 10.4, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 20 mM L-homoarginine) added, gently mixed and incubated at 37°C for 10 min. After adding 97 μl of 1x reaction buffer (100 mM glycine, pH 10.4, 1 mM $MgCl_2$, 1 mM $ZnCl_2$), samples were further incubated at room temperature for 5 min. Three microliters of 4-MUP working solution (diluted 1:10 in reaction buffer from a 10 mM 4-MUP stock solution in DMSO) were added to each sample and incubated for 60 min in the dark at room temperature. Fluorescence was measured with a Spectrafluor Plus multiplate reader (Tecan AG, Hombrechtikon, Switzerland) (excitation 360 nm, emission 460 nm). A standard curve was generated with alkaline phosphatase (14 U/mg protein, Sigma) using 0.1–100 ng per well.

MCP-1 Assays

Bioactivity of MCP-1 protein present in the cellular supernatants obtained from MCP-1–transfected A-10 cells was determined by measuring calcium mobilization in CHO cells stable-transfected with the CCR2b receptor using a fluorometric imaging plate reader (FLIPR) assay. One day before the experiment, 5000 cells per well were plated on 384-well plates (Costar, Bodenheim, Germany). Cells were loaded before measurement with Fluo-4 (0.0002%) for 45 min in culture medium and washed gently four times with HBSS before stimulation. Measurements were recorded as relative fluorescence units after substraction of a negative control measurement and a BIAS subtraction. To determine the amount of bioactive MCP-1 in the supernatants, fluorescence values were referred to a concentration curve performed with recombinant human MCP-1 (Peprotech, Rocky Hill, IL, USA) diluted in A-10–conditioned cell culture medium.

MCP-1 protein concentrations in the supernatants were determined using the commercially available kit OptEIA Human MCP-1 Set (BD Biosciences Pharmingen, Heidelberg, Germany). The assay was performed in 96-well microtiter plates. Microtiter plates were incubated with 100 μl per well of the anti-human MCP-1 capture antibody in carbonate buffer overnight at 4°C and washed three times with a PBS buffer containing Tween-20. Subsequently, 200 μl/well blocking buffer (PBS with 1% bovine serum albumine) were added, incubated for 1 h at room temperature, and plates were washed again as described above before adding the samples. Collected supernatants of the MCP-1 gene transfected cells were diluted, added to the prepared microtiter plates, and incubated for 2 h at room temperature under mild rotation. Following another washing step, biotinylated anti-human MCP-1 antibody (see manufacturer's protocol), avidin-horseradish peroxidase conjugate, and 0.5% BSA-containing Tween buffer were added. After incubation for 1 h at room temperature followed by a washing step, substrate

solution was added, incubated for 15 min in the dark, and the color reaction was stopped by the addition of HCl. OD was measured at 450 nm using an ELISA plate reader.

Statistical Analysis

Statistical analysis was performed using the program WinSTAT for Microsoft Excel. ANOVA (followed by Scheffé's test) was used for comparing the four individual gene transfer formulations; two-sided *t* test was applied comparing differences in size and zeta potential before and after the freeze/thaw cycle. Differences were considered significant for $p < 0.05$.

RESULTS

Biophysical Characterization of Novel Polyplex Formulations

Two different novel stabilized polyplex formulations were tested, one termed DNA/PEG/L-PEI polyplexes, containing DNA complexed with a mixture of the co-polymer PEG-L-PEI (20%) and L-PEI (80%). The other formulation termed DNA/PEG/Mel-PEI consisted of DNA complexed with a mixture of 20% of PEG-L-PEI and 80% of Mel-PEI, which is a conjugate of branched PEI 25-kDa with the membrane active peptide melittin. The ratios of 20%/80% are based on the PEI content of the individual conjugates.

Polyplexes were generated at a DNA concentration of 100 μg/ml in a salt-free buffer (containing 5% w/v glucose to ensure isoosmolarity) to favor the formation of small particles (16). For comparison, a standard formulation of unshielded DNA/L-PEI complexes with known high gene transfer efficiency was generated in the presence of 75 mM salt and 2.5% w/v glucose. Table I shows the biophysical parameters of the polyplexes. The PEG-containing particles were approximately 150 nm in size bearing a near-neutral surface charge.

Table I. Biophysical Properties of Polyplexes

Formulation	Freeze/thaw cycle	Size (nm)	Polydispersity	Zeta potential (mV)
DNA/PEG/	–	101 ± 12**	0.2 ± 0.14**	2.9 ± 0.3
L-PEI	+	256 ± 76**	0.76 ± 0.28**	2.2 ± 0.8
DNA/PEG/	–	138 ± 43*	0.53 ± 0.39	2.2 ± 0.2*
Mel-PEI	+	535 ± 249*	0.92 ± 0.02	12.1 ± 7.7*
DNA/L-PEI	–	1300 ± 200	0.9 ± 0.12	30 ± 10

PEG, polyethylene glycol; L-PEI, linear polyethylenimine; Mel-PEI, melittin-PEI.

DNA/PEG/L-PEI complexes of DNA/PEG/Mel-PEI complexes were generated in HBG (20 mM HEPES, pH 7.1, 5% glucose w/v) with a final concentration of 100 μg/ml DNA at a N/P ratio of 6. This N/P ratio corresponds to 1 μg of DNA (3 nmol of phosphate) complexes with 0.78 μg of PEI (18 nmol of nitrogen). Complex size and zeta potential was measured 30 min after complex formation after freezing the complexes in liquid nitrogen, storage at –20°C for several days, and thawing at 37°C in a water bath. For comparison, standard DNA/L-PEI complexes were generated in 0.5 × HBS (75 mM NaCl, 20 mM HEPES, pH 7.1, 2.5% glucose w/v). Data shown are the mean values of at least three independent experiments ± standard deviation. Statistical significance before and after freeze/thaw cycle at $p < 0.01$ (**) and $p < 0.05$ (*), respectively (*t* test).

In sharp contrast, the standard unshielded DNA/L-PEI complexes are large (>1000 nm) when freshly prepared and carry a high positive surface charge (see also Ref. 17).

Freezing or freeze-drying would be an important strategy for preserving gene transfer formulations based on polycations or cationic lipids (18), but often results in DNA particle aggregation. For this reason, we evaluated the effect of freezing on the biophysical characteristics of our novel polyplexes. Freeze-thawing of the unshielded DNA/L-PEI complexes in the presence or absence of glucose resulted in formation of huge, visible aggregates (10) not applicable for gene transfer. In contrast, with the PEGylated polyplexes after one freeze/thaw cycle, only a moderate but significant increase in particle size to 300 nm (DNA/PEG/L-PEI) and 500 nm (DNA/PEG/Mel-PEI) was observed. The polydispersity index as a measure for the width of the size distribution also increased after the freeze/thaw step. This indicates a mild, partial aggregation of polyplexes to a mixture of small particles and some larger aggregates. Optimization of the cryoformulation medium using different buffer and sugars (19,20) may further improve the particle stability. In the current work, the described cryoconserved formulation in glucose was applied in all further testings.

Cellular Association

Cellular association of DNA/PEG/L-PEI and DNA/L-PEI was studied on A-10 rat smooth muscle cells (Fig. 1). Already low doses (0.5 μg DNA per 100,000 cells) of aggregated, strongly positively charged DNA/L-PEI complexes result in >90% YOYO-1 positive cells (Fig. 1A). In contrast, shielded DNA/PEG/L-PEI particles with a near-neutral surface charge have to be applied at higher doses to reach the same percentage of cells after 4 h of incubation (Fig. 1B). Binding kinetics of both formulations using optimized DNA doses are comparable, with maximum cellular association after 2 h of incubation at 37°C (Figs. 1C–1D).

Toxicity

We studied the effect of different DNA formulations on cellular viability by measuring cellular ATP content (21). DNA/L-PEI complexes were highly toxic even at low doses both on A-10 rat SMCs (Fig. 2A) and PEC's (Fig. 2B). Incorporation of PEG into the polyplex strongly reduced toxicity at all concentrations tested ($p < 0.01$, ANOVA, Scheffé). With PEG-shielded DNA/PEG/L-PEI complexes, toxicity was almost absent at doses up to 0.4 μg DNA per well, and at the maximum dose tested (1.5 μg DNA) 70% (A-10) and >40% (PEC) of the cells were still viable. By contrast, the viability of cells transfected with DNA/L-PEI at this dose was <10%. Similar results as with DNA/PEG/L-PEI treated A-10 cells were obtained with the lipoplex formulation DNA/DC30 (22). Viability of DNA/PEG/Mel-PEI treated cells was slightly lower than DNA/PEG/L-PEI treated cells, but polyplexes were still significantly less toxic compared to DNA/L-PEI. In PEC cells, DNA/PEG/L-PEI and DNA/PEG/Mel-PEI complexes were also significantly less toxic as compared to DNA/L-PEI at all doses tested.

Reporter Gene Expression

Despite the decreased cell binding of PEG shielded polyplexes (see Fig. 1), their transfection potential was, depending

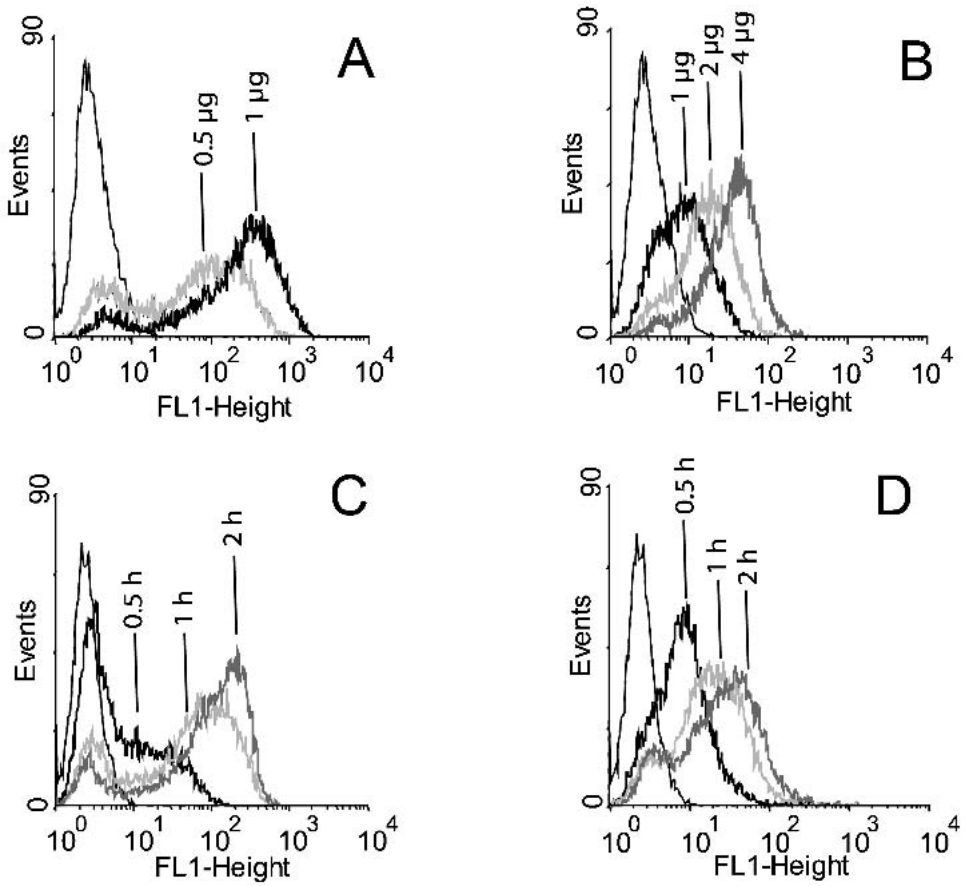

Fig. 1. Cellular association of polyplexes with A-10 vascular smooth muscle cells: 10^5 A-10 cells were plated in 12-well plates 1 day prior to transfection. Plasmid DNA (pAH7-Luc) was labeled with the fluorescent DNA intercalator YOYO-1 prior to complexation as described in "Materials and Methods." Complex formation was carried out as described in Table I. DNA/L-PEI complexes were used fresh 30 min after mixing in 0.5x HBS; DNA/PEG/L-PEI complexes were generated in HBG and used after one freeze/thaw cycle in liquid nitrogen. Cells were incubated with the indicated amount of complexed DNA in 600 µl of medium at 37°C (5% CO_2, humidified atmosphere) and thereafter harvested and analyzed by flow cytometry on a FACScalibur (Becton Dickinson) as described in "Materials and Methods." (A) and (B) Histograms of fluorescence intensities of cells incubated for 4 h at 37°C with different quantities of either (A) DNA/L-PEI complexes or (B) DNA/PEG/L-PEI complexes are shown. Narrow line: untreated cells; full lines: amount of DNA as indicated (0.5 mg, 1 mg, 2 mg, and 4 mg). (C) and (D) Cells were incubated at 37°C for different time periods with either (C) 1 µg DNA/L-PEI complexes or (D) 4 µg DNA/PEG/L-PEI complexes. Narrow line: untreated cells; full lines: incubation time as indicated (0.5 h, 2 h, and 4 h).

on the cells used, equal or even increased compared to aggregated DNA/L-PEI complexes (Fig. 3). Highest gene expression was achieved in A-10 cells with DNA/PEG/Mel-PEI complexes, resulting in a 5-fold increase as compared to unshielded DNA/L-PEI complexes. DNA/PEG/L-PEI complexes were equal in their transfection efficiency to DNA/L-PEI polyplexes in A-10 cells, whereas in PSMCs or endothelial cells, increased doses of PEGylated polyplexes were needed to achieve a similar luciferase activity.

To determine the percentage of transfected cells, EGFP was used as a reporter gene (Table II). Again, DNA/PEG/Mel-PEI complexes were most efficient, transfecting almost 50% of cells with EGFP, compared to DNA/L-PEI and DNA/PEG/PEI resulting in 22% and 17% EGFP positive cells, respectively. Using optimized DNA doses for the individual formulations, the activity of secreted alkaline phosphatase (SEAP) was measured in the supernatants of SEAP plasmid transfected A-10 cells (Fig. 4). For all formulations tested, maximum gene expression rate was obtained 3–5 days after transfection. Such a time course for a secreted protein is different from the expression kinetics for intracellular luciferase, where the maximum gene expression rate was observed after 24 h (M.O., unpublished data). Both PEG-shielded formulations resulted in an up to 4-fold increase in expression rate as compared to DNA/L-PEI, and the total amount of SEAP activity produced within 9 days was 4- to 5-fold higher (190 ng for DNA/L-PEI, 800 ng for DNA/PEG/L-PEI, and 960 ng for DNA/PEG/Mel-PEI per 10,000 cells seeded).

Finally, the ability of smooth muscle cells to express functional secreted MCP-1 protein was tested after transfection of a MCP-1 encoding plasmid under the control of a CMV promoter (Fig. 5). Significant levels of MCP-1 were detected for all transfection formulations used. Up to 125 ng/ml active MCP-1 was found 72 h after transfection per ml supernatant (Fig. 5A),

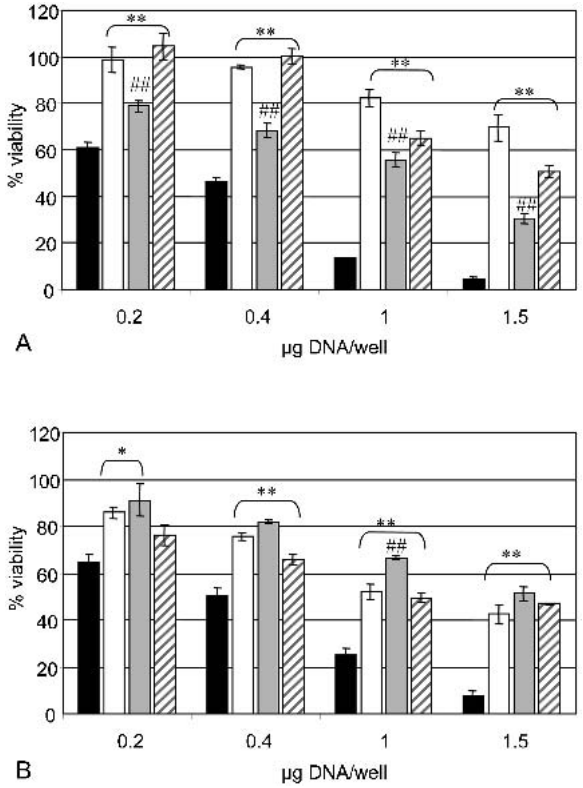

Fig. 2. Viability test for smooth muscle cells and endothelial cells. The day before transfection, (A) A-10 cells or (B) primary porcine endothelial cells (PEC) were seeded in 96-well plates (white, with clear bottom) at 10^5 cells per well. Cells were transfected in 100 µl mediums with the indicated amount of complexed firefly luciferase encoding plasmid. PEG containing polyplexes were generated in HBG at a final DNA concentration of 100 µg/ml and subjected to one freeze/thaw cycle prior to transfection. DNA/L-PEI complexes were generated in 0.5x HBS at a final DNA concentration of 20 µg/ml. DNA/DC30 lipoplexes were reconstituted from a lyophilisate (final DNA concentration, 25 µg/ml) and used thereafter for transfection. After an incubation period of 4 h, fresh medium was added to yield a final volume of 200 µl, and 24 h after transfection the cellular ATP content was measured as described in "Materials and Methods." Non-transfected cells were used as a 100% viability control. Black bars: DNA/L-PEI; white bars: DNA/PEG/L-PEI; gray bars: DNA/PEG/Mel-PEI; hatched bars: DNA/DC30. Cells were incubated with increasing amounts of formulated DNA ranging from 0.2, 0.4, 1.0 to 1.5 µg/ml. Mean values from triplicates ± standard deviation are shown. Statistical significance vs. DNA/L-PEI at $p < 0.01$ (**) or $p < 0.05$ (*); statistical significance DNA/PEG/Mel-PEI vs. DNA/PEG/L-PEI at $p < 0.05$ (#) or $p < 0.01$ (##).

whereas the MCP-1 protein content in cellular supernatants was up to 400 ng per ml (Fig. 5B). This discrepancy could be explained by a partial loss of MCP-1 bioactivity during 72 h of continuous incubation at 37°C in the presence of serum.

DISCUSSION

A gene transfer formulation based on polyethylenimine (PEI) was developed for efficient transfection of vascular smooth muscle cells (SMCs) and endothelial cells (ECs). The incorporation of PEG as a co-polymer with PEI physically stabilized the polyplex formulation, enabled storage in frozen form without loss of bioactivity, and strongly reduced the cellular toxicity of PEI polyplexes.

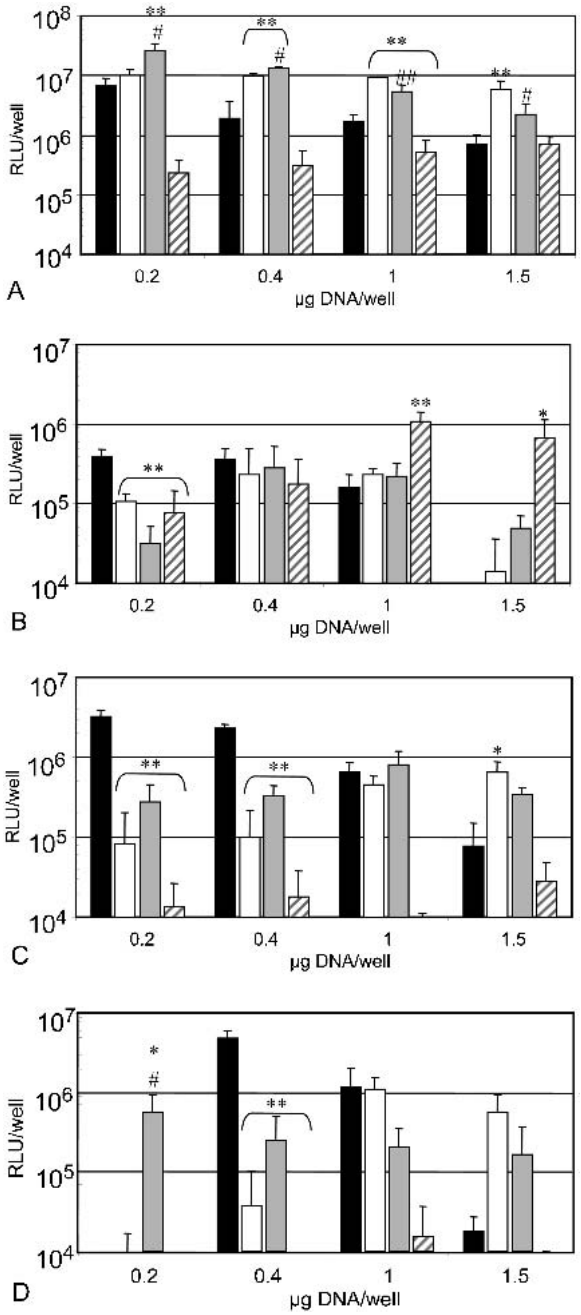

Fig. 3. Luciferase reporter gene expression on smooth muscle cells and endothelial cells. (A) A-10 cells, (B) PSMC, (C) PEC, and (D) HUVEC cells were seeded in 96-well plates at densities of 10^5 cells per well and transfected as described in Fig. 2. Cells were incubated with increasing amounts of formulated pAH7-Luc DNA ranging from 0.2, 0.4, and 1.0 to 1.5 µg/ml using stock solutions of complexes. Black bars: DNA/L-PEI; white bars: DNA/PEG/L-PEI; gray bars: DNA/PEG/Mel-PEI; hatched bars: DNA/DC30. After an incubation period of 4 h, fresh medium was added to a final volume of 200 µl. At 24 h after transfection, luciferase activity was measured as described (12). Mean values from triplicates ± standard deviation are shown. Statistical significance vs. DNA/L-PEI at $p < 0.01$ (**) or $p < 0.05$ (*); statistical significance DNA/PEG/Mel-PEI vs. DNA/PEG/L-PEI at $p < 0.05$ (#) or $p < 0.01$ (##).

Table II. Enhanced Green Fluorescent Protein Reporter Gene Expression on Smooth Muscle Cells

Formulation	% EGFP positive cells
Control	0.11 ± 0.01
DNA/PEG/ L-PEI	17.2 ± 6.5
DNA/PEG/ Mel-PEI	47.4 ± 3.8*
DNA/L-PEI	22.1 ± 0.4

PEG, polyethylene glycol; L-PEI, linear polyethylenimine; Mel-PEI, melittin-PEI.

A-10 cells (60,000 cells plated 1 day before transfection, 24-well plate) were transfected with 3.6 μg EGFP encoding plasmid per well in 600 μl medium. After 4 h, 1 ml fresh medium was added. Cells were harvested after total 24 h and assayed for EGFP expression as described (16). Mean values from duplicates ± standard deviation are shown.

* Statistical significance DNA/PEG/Mel-PEI vs. DNA/PEG/L-PEI and DNA/L-PEI $p < 0.05$ (ANOVA, plus Scheffé's test).

Co-polymers of cationic polymers with hydrophilic polymers such as PEG have been evaluated as transfection reagents by several research groups (e.g., see Refs. 9–11, 23–26), demonstrating their beneficial characteristics such as high physical stability and low toxicity both in cell culture and *in vivo*. The incorporation of PEG into polyplexes is not without pitfalls; their use can result in reduced transfection efficiency as compared to non-PEGylated polyplexes (9,23). At least three major reasons have to be considered as explanation for this finding.

First, a high content of PEG in the transfection complex may hinder proper condensation of DNA and particle formation (24,27). One way to avoid this problem is "post-PEGylation," as the subsequent introduction of PEG or other hydrophilic polymers into already formed DNA complexes does not interfere with DNA condensation (28). Alternatively, as applied in our current work, only a fraction of total PEI (20% in the current study) in the polyplex is used in PEG-modified form. Our recent studies demonstrated that careful titration of PEG content provides shielding and stabilization and maintains high transfection efficiency (10).

Second, incorporation of PEG into the complexes may significantly reduce cellular association (29). This was also observed in the current investigation, but as shown here can be compensated by increasing the polyplex concentration as shown in Fig. 1.

Third, incorporation of PEG might reduce endosomal escape of DNA complexes (16,30,31). This step can be very limiting with small polyplexes (16), whereas larger PEGylated particles show better transfection characteristics (10,30). To evaluate the aspect of endosomal escape, we incorporated a synthetic melittin peptide into the PEG-shielded polyplexes. This was based on the rationale that melittin can enhance the intracellular release of internalized polyplexes from endosomes into the cytoplasm, as previously reported for non-shielded polyplexes (12). Indeed, a significant increase of reporter gene expression was observed using DNA/PEG/Mel-PEI in three of the four cell types evaluated (Figs. 3A, 3C, and 3D), and this effect was most prominent at low DNA doses.

A key element for gene therapy applications is the lack of toxicity. PEGylation can contribute here in dual fashion: it reduces toxicity both on the host organism level and on the cellular level. The low cellular toxicity obtained with PEG-shielded polyplexes as observed in this study (Fig. 2) is in good agreement with recently published data, where similar

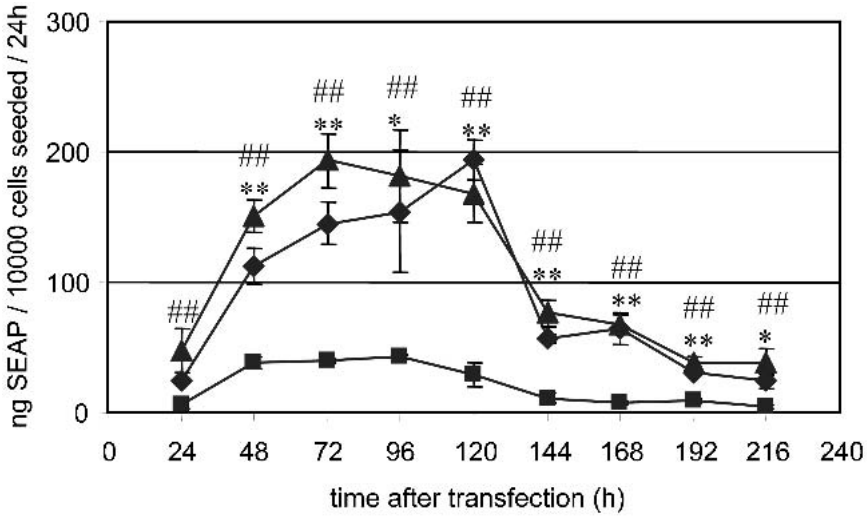

Fig. 4. Secreted alkaline phosphatase (SEAP) reporter gene expression on smooth muscle cells A-10 cells were transfected in 96-well plates analogously as described in Fig. 3 using SEAP as reporter gene. Cells were transfected with the indicated formulation at the optimal DNA dose (determined in a separate experiment measuring SEAP activity 48 h after transfection), medium was replaced every 24 h, and SEAP activity was assayed in the supernatants. Squares: DNA/L-PEI in 0.5x HBS, 25 ng DNA/well; diamonds: DNA/PEG/L-PEI in HBG, 400 ng DNA/well; triangles: DNA/PEG/Mel-PEI in HBG, 100 ng DNA/well. Mean values from triplicates ± standard deviation are shown. Statistical significance DNA/PEG/L-PEI vs. DNA/L-PEI at $p < 0.01$ (**) or $p < 0.05$ (*); statistical significance DNA/PEG/Mel-PEI vs. DNA/L-PEI at $p < 0.05$ (#) or $p < 0.01$ (##).

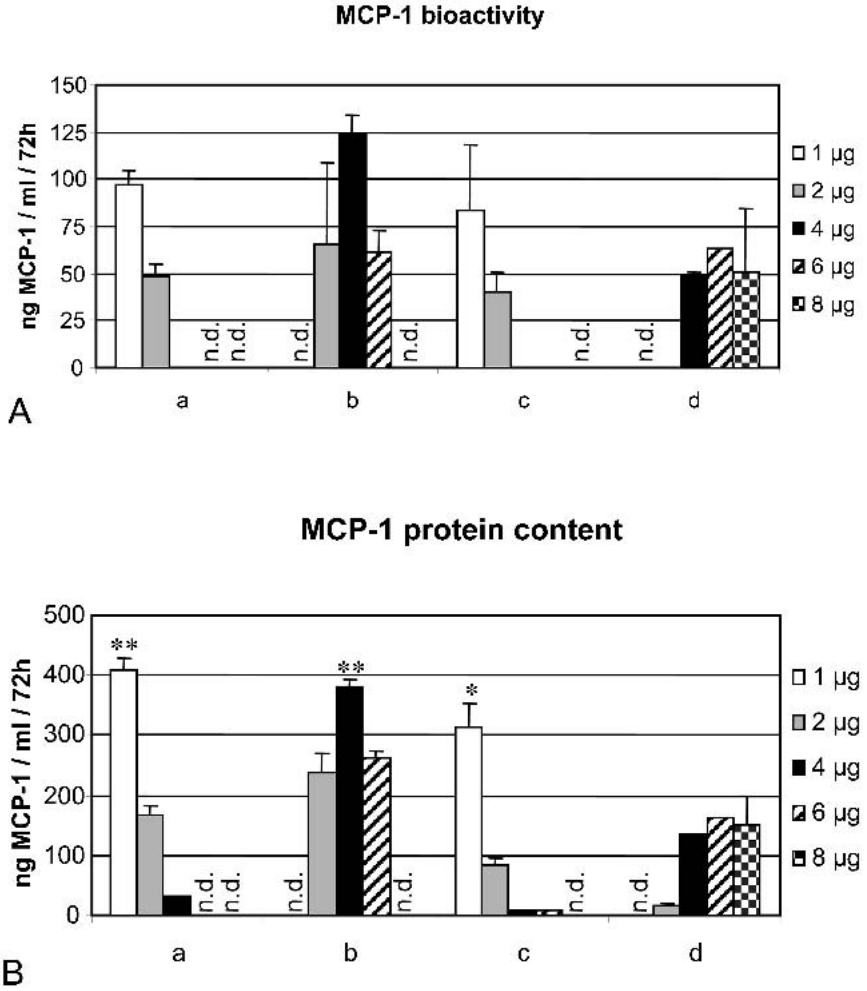

Fig. 5. Expression of MCP-1 protein in transfected A-10 smooth muscle cells. A-10 cells (50,000 cells plated 1 day before transfection, 12-well plate) were transfected with the indicated amount of MCP-1 encoding plasmid pAH7-MCP1 per well in 1 ml medium. After 72 h, supernatants were removed, frozen in liquid nitrogen, and stored for further analysis. Complexes were generated as described in Fig. 3. (A) Bioactivity of expressed MCP-1 protein; (B) MCP-1 protein content. (a) DNA/L-PEI in 0.5x HBS; (b) DNA/PEG/L-PEI in HBG; (c) DNA/PEG/Mel-PEI in HBG; (d) DNA/DC30. Mean values from triplicates ± standard deviation are shown. n.d.: not done. Statistical analysis (ANOVA, Scheffé's test) was carried out comparing the optimal DNA doses of the individual formulations used for transfection. Statistical significance vs. DNA/DC30 at $p < 0.01$ (**) or $p < 0.05$ (*).

effects were found with PEG-PEI conjugates (9,25). The cytotoxicity of standard PEI polyplexes presumably has its cause in a strong, unspecific interaction of the highly positive charged surface of the PEI polyplexes with cellular membranes; this can be blocked effectively by PEG. The function of PEG in reducing unspecific interactions is also valuable for *in vivo* applications. PEG blocks the interaction with blood components and nontarget cells, and in this manner strongly reduces *in vivo* toxicity of PEI polyplexes (11,30). A very similar PEG/PEI formulation as described in the current paper was successfully applied systemically in mice for targeted expression in distant, subcutaneous tumors without any sign of systemic toxicity (10).

Despite shielding of particles from unspecific interactions, the transfection potential of PEG containing polyplexes (both DNA/PEG/L-PEI and DNA/PEG/Mel-PEI) in the SMC cell line A-10 was similar or even increased at all DNA concentrations as compared to standard DNA/L-PEI polyplexes (Fig. 3A). Up to approximately 50% of transfected cells expressed a EGFP marker gene (Table II) and up to 200 ng SEAP/10,000 cells/24 h were produced by DNA/PEG/Mel-PEI polyplexes (Fig. 4). In primary SMC or endothelial cells, at low DNA dosage a lower expression was observed with the PEGylated polyplexes, but increasing DNA doses also recovered reporter gene expression (Fig. 3).

Stability of DNA formulation is another important pharmaceutical aspect. All transfection results with PEGylated polyplexes presented in this study were obtained by using cryoconserved polyplex formulations. After the freeze/thaw step, a moderate increase in particle size was observed (Table I). Optimization of the cryoformulation medium may further improve the particle stability (20). The slight changes in particle size in the current glucose formulation is not considered to be critical, as the biological gene transfer activity of PEG-

shielded polyplexes was not negatively affected. This is in line with our earlier observation that moderate aggregation of PEGylated polyplexes retains or even enhances the transfection activity (10).

In summary, polyethylenimine-based polyplexes were developed that display reduced toxicity, can be cryoconserved, and efficiently transfect cells of the vasculoendothelial system. Transfection with a therapeutic plasmid encoding MCP-1 resulted in expression and secretion of bioactive MCP-1, with the most biocompatible formulation DNA/PEG/L-PEI expressing the highest amount of bioactive MCP-1. In this regard, the current study suggests the possible use of a PEGylated polyplex formulation for the local vascular gene expression of MCP-1 as a gene therapeutic approach for arterial occlusion disease. The time frame of gene expression observed by the formulation (several days after a single transfection) should be sufficient for a therapeutic effect of MCP-1 locally secreted *in vivo*. Voskuil *et al.* compared continuous local infusion of MCP-1 protein after ligation of the femoral artery in minipigs either for 3 days or 2 weeks (3). For both application schemes, a similar outcome was observed suggesting that the therapeutic effect of MCP-1 is most pronounced within the first days after arterial occlusion. Due to the nonviral and nontoxic character, the gene transfer system presented in our study offers a high degree of biological safety. Even in the case of inadvertent systemic leakage of the polyplexes after local catheter-mediated application, no significant transgene expression in nontarget tissues would be expected, as PEG-shielded DNA/L-PEI polyplexes applied systemically revealed a very low toxicity and low transfection potential for healthy organs (10,11). Additional safety may be obtained by using a tissue-specific promoter for smooth muscle cells (32).

ACKNOWLEDGMENTS

Dr. Klaus Bergemann is greatly acknowledged for the ELISA-based determination of MCP-1. Wolfgang Rödl is acknowledged for his excellent technical assistance in complex formulation and transfection experiments. Thomas Zieher is acknowledged for his expert technical assistance in the determination of MCP-1 bioactivity. We are grateful to Dr. Greg Walker and Dr. Carsten Culmsee for careful review of the manuscript and help with statistical analysis.

REFERENCES

1. D. Scholz, W. J. Cai, and W. Schaper. Arteriogenesis, a new concept of vascular adaptation in occlusive disease. *Angiogenesis* **4**:247–257 (2001).
2. P. Carmeliet. Mechanisms of angiogenesis and arteriogenesis. *Nat. Med.* **6**:389–395 (2000).
3. M. Voskuil, N. van Royen, I. E. Hoefer, R. Seidler, B. D. Guth, C. Bode, W. Schaper, J. J. Piek, and I. R. Buschmann. Modulation of collateral artery growth in a porcine hindlimb ligation model using MCP-1. *Am. J. Physiol. Heart Circ. Physiol.* **284**: H1422–H1428 (2003).
4. I. E. Hoefer, N. van Royen, I. R. Buschmann, J. J. Piek, and W. Schaper. Time course of arteriogenesis following femoral artery occlusion in the rabbit. *Cardiovasc. Res.* **49**:609–617 (2001).
5. G. Zuber, E. Dauty, M. Nothisen, P. Belguise, and J. P. Behr. Towards synthetic viruses. *Adv. Drug Deliv. Rev.* **52**:245–253 (2001).
6. E. Wagner. Strategies to improve DNA polyplexes for in vivo gene transfer – will ′artificial viruses′ be the answer? *Pharm. Res.* **21**:8–14 (2004).
7. W. T. Godbey, K. K. Wu, and A. G. Mikos. Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability. *Biomaterials* **22**:471–480 (2001).
8. P. Chollet, M. C. Favrot, A. Hurbin, and J. L. Coll. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J. Gene Med.* **4**:84–91 (2002).
9. A. Kichler, M. Chillon, C. Leborgne, O. Danos, and B. Frisch. Intranasal gene delivery with a polyethylenimine-PEG conjugate. *J. Controll. Rel.* **81**:379–388 (2002).
10. M. Kursa, G. F. Walker, V. Roessler, M. Ogris, W. Roedl, R. Kircheis, and E. Wagner. Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. *Bioconjug. Chem.* **14**:222–231 (2003).
11. M. Ogris, G. Walker, T. Blessing, R. Kircheis, M. Wolschek, and E. Wagner. Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J. Controll. Rel.* **91**:173–181 (2003).
12. M. Ogris, R. C. Carlisle, T. Bettinger, and L. W. Seymour. Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. *J. Biol. Chem.* **276**:47550–47555 (2001).
13. J. Pelisek, S. Armeanu, and S. Nikol. Quiescence, cell viability, apoptosis and necrosis of smooth muscle cells using different growth inhibitors. *Cell Prolif.* **34**:305–320 (2001).
14. M. Ogris, P. Steinlein, S. Carotta, S. Brunner, and E. Wagner. A versatile assay to study cellular uptake of gene transfer complexes by flow cytometry. *Biochim. Biophys. Acta* **1474**:237–243 (2000).
15. K. R. Gee, W. C. Sun, M. K. Bhalgat, R. H. Upson, D. H. Klaubert, K. A. Latham, and R. P. Haugland. Fluorogenic substrates based on fluorinated umbelliferones for continuous assays of phosphatases and beta-galactosidases. *Anal. Biochem.* **273**:41–48 (1999).
16. M. Ogris, P. Steinlein, M. Kursa, K. Mechtler, R. Kircheis, and E. Wagner. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther.* **5**:1425–1433 (1998).
17. L. Wightman, R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa, and E. Wagner. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **3**:362–372 (2001).
18. T. J. Anchordoquy, S. D. Allison, M. C. Molina, L. G. Girouard, and T. K. Carson. Physical stabilization of DNA-based therapeutics. *Drug Discovery Today* **6**:463–470 (2001).
19. H. Talsma, J. Y. Cherng, H. Lehrmann, M. Kursa, M. Ogris, W. E. Hennink, M. Cotten, and E. Wagner. Stabilization of gene delivery systems by freeze-drying. *Int. J. Pharm.* **157**:233–238 (1997).
20. J. Y. Cherng. P. vd Wetering, H. Talsma, D. J. Crommelin, and W. E. Hennink. Stabilization of polymer-based gene delivery systems. *Int. J. Pharm.* **183**:25–28 (1999).
21. S. P. Crouch, R. Kozlowski, and K. J. Slater, and J. Fletcher. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods* **160**:81–88 (1993).
22. K. Fife, M. Bower, R. G. Cooper, L. Stewart, C. J. Etheridge, R. C. Coombes, L. Buluwela, and A. D. Miller. Endothelial cell transfection with cationic liposomes and herpes simplex-thymidine kinase mediated killing. *Gene Ther.* **5**:614–620 (1998).
23. H. K. Nguyen, P. Lemieux, S. V. Vinogradov, C. L. Gebhart, N. Guerin, G. Paradis, T. K. Bronich, V. Y. Alakhov, and A. V. Kabanov. Evaluation of polyether-polyethyleneimine graft copolymers as gene transfer agents. *Gene Ther.* **7**:126–138 (2000).
24. P. Erbacher, T. Bettinger, P. Belguise-Valladier, S. Zou, J. L. Coll, J. P. Behr, and J. S. Remy. Transfection and physical properties of various saccharide, poly(ethylene glycol), and antibody-derivatized polyethylenimines (PEI). *J. Gene Med.* **1**:210–222 (1999).
25. K. Kunath, A. von Harpe, H. Petersen, D. Fischer, K. Voigt, T. Kissel, and U. Bickel. The structure of PEG-modified poly(ethylene imines) influences biodistribution and pharmacokinetics of their complexes with NF-kappaB decoy in mice. *Pharm. Res.* **19**:810–817 (2002).
26. T. Merdan, J. Callahan, H. Petersen, K. Kunath, U. Bakowsky, P. Kopeckova, T. Kissel, and J. Kopecek. Pegylated polyethylenimine-fab′ antibody fragment conjugates for targeted gene de-

livery to human ovarian carcinoma cells. *Bioconjug. Chem.* **14**: 989–996 (2003).
27. M. A. Wolfert, E. H. Schacht, V. Toncheva, K. Ulbrich, O. Nazarova, L. W. Seymour, V. Toncheva, M. A. Wolfert, P. R. Dash, D. Oupicky, K. Ulbrich, L. W. Seymour, and E. H. Schacht. Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Hum. Gene Ther.* **7**:2123–2133 (1996).
28. P. R. Dash, M. L. Read, K. D. Fisher, K. A. Howard, M. Wolfert, D. Oupicky, V. Subr, J. Strohalm, K. Ulbrich, and L. W. Seymour. Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. *J. Biol. Chem.* **275**:3793–3802 (2000).
29. M. Ogris, P. Steinlein, S. Carotta, S. Brunner, and E. Wagner. DNA/polyethylenimine transfection particles: Influence of ligands, polymer size, and PEGylation on internalization and gene expression. *AAPS. PharmSci.* **3**:1–11 (2001).
30. M. Ogris, S. Brunner, S. Schuller, R. Kircheis, and E. Wagner. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* **6**:595–605 (1999).
31. M. Keller, R. P. Harbottle, E. Perouzel, M. Colin, I. Shah, A. Rahim, L. Vaysse, A. Bergau, S. Moritz, C. Brahimi-Horn, C. Coutelle, and A. D. Miller. Nuclear localisation sequence templated nonviral gene delivery vectors: investigation of intracellular trafficking events of LMD and LD vector systems. *Chembiochem.* **4**:286–298 (2003).
32. M. C. Keogh, D. Chen, J. F. Schmitt, U. Dennehy, V. V. Kakkar, and N. R. Lemoine. Design of a muscle cell-specific expression vector utilizing human vascular smooth muscle alpha-actin regulatory elements. *Gene Ther.* **6**:616–628 (1999).