Pharmaceutical Research, Vol. 24, No. 12, December 2007 (© 2007)
DOI: 10.1007/s11095-007-9403-4

Research Paper

Novel Biocompatible Cationic Copolymers Based on Polyaspartylhydrazide Being Potent as Gene Vector on Tumor Cells

Manfred Ogris,[1,4] Arun K. Kotha,[1,3] Nicole Tietze,[1] Ernst Wagner,[1] Fabio Salvatore Palumbo,[2] Gaetano Giammona,[2] and Gennara Cavallaro[2,5]

Received March 1, 2007; accepted July 2, 2007; published online 16 October 2007

Introduction. The reaction between α,β-poly(aspartylhydrazide) (PAHy), a water soluble synthetic polymer and 3-(carboxypropyl)trimethyl-ammonium chloride (CPTACl) produced copolymers bearing permanent positive charges (PAHy–CPTA) with molecular weight of 10 kDa and PAHy–CPTA copolymers differing in positive charge amount (18–58%) were chosen for biological investigations.
Materials and methods. Biophysical properties of DNA/PAHy–CPTA polyplexes were evaluated in terms of DNA condensation, zeta potential and size distribution. Cytotoxicity studies on Neuro2A murine neuroblastoma cells evidenced absence of toxicity of these copolymers up to 300 μg/ml unlike linear polyethylenimine (LPEI) that was highly toxic already at 20 μg/ml.
Results and Discussion. PAHy–CPTA copolymers did not induce any erythrocyte aggregation up to 1 mg/ml. Cellular interaction studies of PAHy–CPTA polyplexes evidenced a faster binding of these polyplexes with cells compared to DNA/LPEI polyplexes. The *in vitro* transfection ability of PAHy–CPTA polyplexes was strongly affected by experimental conditions reaching about 10% of the transfection efficiency of optimized LPEI polyplexes.
Conclusions. Finally, *in vivo* application studies confirmed the biocompatibility of PAHy–CPTA copolymers. With LPEI, clear signs of microvesicular fatty liver were observed and with LPEI polyplexes significant weight loss. In strong contrast, PAHy–CPTA did not induce histopathological changes or weight loss.

KEY WORDS: cytotoxicity; liver toxicity; nonviral gene delivery; transfection.

INTRODUCTION

Synthetic gene delivery systems offer the possibility of safe and repeated administration of transgenes. A promising type of non-viral vector is a positively charged polymer that is able to condense the polyanionic DNA macromolecules to form so called polyplexes (1). In principle the polycation should be non toxic as well as easy to synthesize and able to compact DNA into nanoparticles small enough to extravasate and to enter into target cells (2). Despite several interesting results, many polycations can not be used due to their high toxicity after systemic application (3). This points out the importance for a biocompatible, non toxic carrier polymer, which can be easily modified in terms of amount of positive charge as well as molecular weight. Moreover, important properties of synthetic gene delivery systems are a high hydrophilicity, avoidance of RES capture, low net positive charge to reduce the clearance from circulation and at the same time, high physical stability i.e. low tendency to aggregate. For example, Howard *et al.* (4) examined the effect of increasing the hydrophilicity of the cationic polymers, based on quaternary ammonium groups, on the DNA complexing ability, showing that an appropriate balance of hydrophilicity and physical stability are key aspects of effective gene delivery vectors.

α,β-polyaspartylhydrazide (PAHy) is a water soluble, non-toxic, non-antigenic and non immunogenic polymer, obtained from polysuccinimide by reaction with hydrazine (5). Besides these favorable pharmacological properties, this polymer shows a good chemical reactivity due to the presence of hydrazinic groups (one for each repeating unit). This allows simple insertion of molecules including drugs or positively charged groups into its structure (6–8).

Cationic derivatives of polyaspartylhydrazide, with different amount of positive charges, were prepared by reaction with glycidyltrimethylammonium chloride (PAHy–GTA) and these materials showed interesting potential for use as systemic DNA carriers because of their relatively low toxicity, absence of accumulation in the liver, good DNA complexing and transfecting abilities (7). However, the reaction between PAHy and GTA was not efficient enough mainly in terms of molecular weight of resulting copolymers.

[1] Pharmaceutical Biology-Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität, Butenandtstr. 5-13, 81377, Munich, Germany.
[2] Dipartimento di Chimica e Tecnologie Farmaceutiche, University of Palermo, Via Archirafi 32, 90123, Palermo, Italy.
[3] Institute for Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany.
[4] To whom correspondence should be addressed. (e-mail: manfred.ogris@cup.uni-muenchen.de)
[5] To whom correspondence should be addressed. (email: gennacav@unipa.it)

Therefore we prepared different PAHy based polycations with easily modulable positive charge amounts by the reaction of PAHy with carboxypropyltrimethylammonium chloride (CPTA) (PAHy–CPTA) to obtain proper molecular weights of the resulting polycations. Preliminary physicochemical characterization studies on polyplexes obtained with DNA (from calf thymus) evidenced the notable properties of these polycations as gene delivery systems (8). In this study it was shown that PAHy–CPTA copolymers efficiently retarded calf thymus DNA and protected the DNA from nuclease degradation.

In the present paper a biological study on four PAHy–CPTA polycations differing in positive charge amounts (18, 33, 45 and 58 mol% respectively) and in relative hydrophilicity has been performed including biophysical evaluation of polyplexes, cell interaction, *in vitro* transfection as well as *in vivo* application.

MATERIALS AND METHODS

Materials

α,β-poly(aspartylhydrazide)-3-(carboxypropyl) trimethyl ammonium chloride was synthesized with different degrees of derivatisation (DD% molar percent) and molecular weights as described (8) and used as a 10 mg/ml stock solution dissolved in water.

Linear PEI with an average molecular weight of 22 kDa (LPEI) was synthesized and used as described recently (9). Branched PEI 25 kDa (BPEI) was purchased from Sigma-Aldrich (Munich, Germany) and used as a 1 mg/ml stock solution neutralized with HCl. Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) was propagated in *Escherichia coli* and for *in vitro* studies purified with the Qiagen EndoFree GigaKit (Qiagen, Hilden, Germany) as described (10). For *in vivo* application pCMVLuc was produced by Plasmid Factory (Plasmid Factory, Bielefeld, Germany) with an endotoxin content<1 EU/mg DNA. pCMVLuc was covalently labeled with the fluorophores Cy3 or Cy5 using the Label IT kits (MIRUS, Madison, WI) as described by Boeckle *et al.* (10). All other reagents were purchased from Sigma-Aldrich (Munich, Germany).

Polyplex Formation

Polyplexes were generated by diluting DNA and PAHy–CPTA copolymers in different buffers such as HBG (20 mM HEPES pH 7.1, 5% glucose *w/v*), HBS (20 mM HEPES pH 7.1, 150 mM NaCl) or HBS1/2 (20 mM HEPES pH 7.1, 2.5% glucose w/v, 75 mM NaCl) at indicated *w/w* or molar ratios of amine/phosphate (N/P). Plasmid DNA was diluted to a final concentration of 40 μg/ml (for *in vitro* studies) respectively 400 μg/ml (for *in vivo* studies). The polymer solution was diluted in the same buffer to a similar volume. PAHy–CPTA solution was added to the DNA solution followed by rapid up-down pipetting (flash mixing) to ensure a homogenous complex formation. For *in vitro* studies, polyplexes were prepared at a final DNA concentration 20 μg/ml, for *in vivo* application at 200 μg/ml. DNA/LPEI polyplexes were prepared at a molar ratio of PEI nitrogen to DNA phosphate (N/P) of 6 (in HBS1/2 for *in vitro* studies, in HBG for *in vivo* application). The N/P ratio for PAHy–CPTA/DNA polyplexes denotes the molar ratio of quaternary amines (in the CPTA group) to phosphate in DNA.

Measurement of Particle Size and Zeta Potential

Particle size and zeta potential of polyplexes was measured using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK) as described recently (10). For estimation of the surface charge, complexes were further diluted with 10 mM NaCl solution to obtain a final DNA concentration of 4 μg/ml.

Ethidium Bromide Exclusion Assay

Aliquots of PAHy–CPTA with different DD% or LPEI were added sequentially to a DNA solution (20 μg/ml) in the indicated buffer containing 400 ng/ml ethidium bromide (EtBr) and the decrease in fluorescence measured in a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Australia). The EtBr/DNA fluorescence (λex 510 nm, λem 590 nm) was set to 100% prior to addition of polycation.

Cell Culture

Neuro2A (murine neuroblastoma; ATCC CCl-131) cells were cultured in DMEM (Biochrom, Berlin, Germany) supplemented with 10% FCS. HuH7 (human hepatocellular carcinoma; JCRB 0403; Tokyo, Japan) were cultured in DMEM high glucose/F12 (1/1) supplemented with 10% FCS.

MTT Assay

Neuro2A cells were incubated with indicated amounts of polycations in 100 μl DMEM/FCS. After 48 h of incubation medium was removed and 200 μl of fresh medium containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) was added. Plates were kept for 3 h at 37°C. During this period MTT is converted into a colored, water insoluble formazan salt by

Table I. Molecular Parameters of PAHy–CPTA Copolymers

	Y	Mw (kDa)	Mw/Mn	DD%	Mw/pos charge	Yield %
PAHy–CPTA	0.25	11.4	1.21	18±2	877	97
	0.5	11.1	1.20	33±2	616	92
	1.0	9.9	1.27	48±3	450	93
	1.5	9.9	1.28	58±3	413	96
PAHy		14.5	1.50			

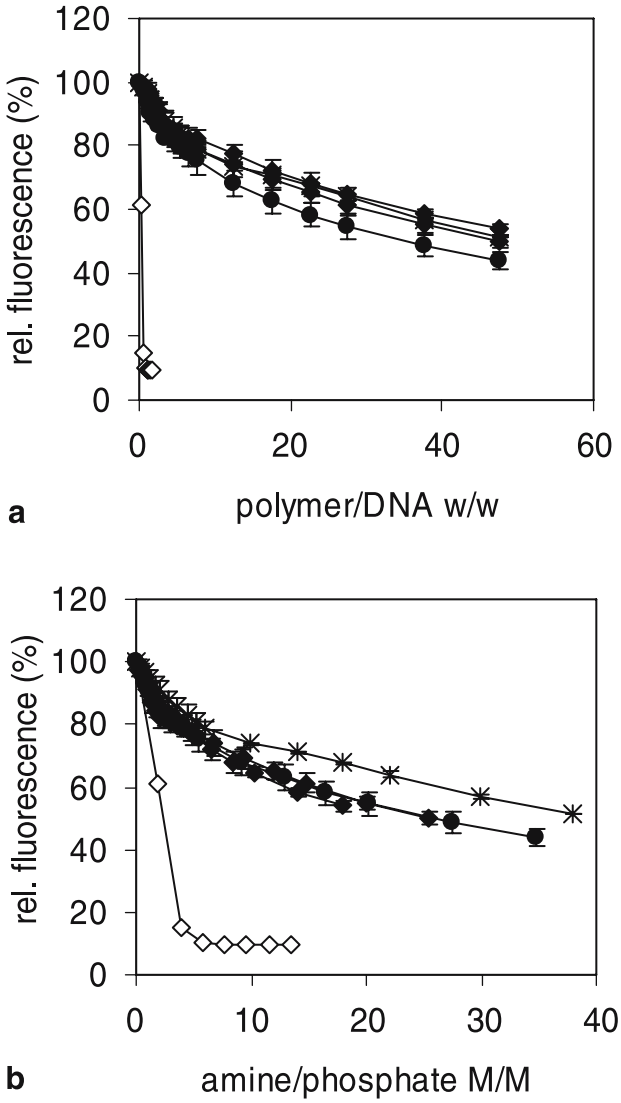

Fig. 1. Condensation of plasmid DNA by PAHy–CPTA copolymers. DNA was diluted to 40 μg/ml in HBG buffer containing 400 ng/ml Et-Br and fluorescence set to 100% as described in "MATERIALS AND METHODS." Indicated polymer solutions were added stepwise and fluorescence measured. Measurements were carried out in triplicates; mean values±standard deviation are shown. Et-Br fluorescence is plotted *vs*. polymer/DNA *w*/*w* ratio (**a**) or molar ratios of amine (for PEI: primary, secondary and tertiary, for PAHy–CPTA quaternary) to phosphate in pDNA (**b**). *Open diamond* LPEI; *square* PAHy–CPTA18; *diamond* PAHy–CPTA33; *circle* PAHy–CPTA48; *cross* PAHy–CPTA58

the metabolic activity of viable cells. Medium was removed from the plates and the cells frozen at −80°C. After thawing the cells 130 μl of DMSO solution was added to dissolve the insoluble formazan salt and absorbance was measured with a plate reader (Tecan, Grödig, Austria) at 590 nm deducting reference absorbance at 630 nm.

Erythrocyte Aggregation

Erythrocytes were obtained from fresh citrated mouse blood, separated from plasma by centrifugation and washed several times with HBG buffer. 50 μl of the erythrocyte suspension was diluted with 250 μl HBG buffer containing 10–100 μg of polymer and incubated at room temperature in a 24-well plate for up to 20 minutes. Erythrocytes were viewed with a Zeiss Axiovert-200 microscope (Carl Zeiss, Oberkochen, Germany) after further dilution of the suspension (1:10 in HBG buffer) and pictures were taken with a digital camera (Sony).

Luciferase Reporter Gene Assay

Cells were seeded in 96 well plates (TPP, Trasadingen, Switzerland) at a density of 5,000 cells per well 24 h prior to transfection. Transfection complexes prepared at different polymer/(pCMVLuc) weight or charge ratios and added to the cells in 100 μl fresh culture medium with FCS. Culture medium was either changed 4 h after transfection and replaced by 100 μl fresh culture medium; alternatively polyplexes were kept on the cells until analysis of reporter gene expression. Luciferase activity was measured after 24 or 48 h as described recently (10). Transfection efficiency was expressed as relative light units (RLU) per seeded cells. Two ng of recombinant luciferase (Promega, Mannheim, Germany)) correspond to 10^7 light units (RLU).

Epifluorescence Microscopy

Cells were seeded in Lab-Tek 8 Chambered Coverglasses (Nalge Nunc International, Naperville, IL, USA) at a density of 10^4 cells per chamber 24 h prior to transfection. Polyplexes were generated using 4% Cy3-labeled DNA and cells were transfected at a concentration of 250 ng DNA per well in 200 μl FCS containing medium. After a 24 h incubation period at 37°C polyplex containing medium was exchanged by fresh medium and live cells were viewed on an Axiovert 200 fluorescence microscope equipped with a Zeiss Axiocam camera. Light was collected through a 63×1.4 NA oil immersion objective. Cy3 fluorescence was excited using a 546/12 nm bandpass filter and emission was detected using a 575–640 nm bandpass filter. Transmitted light images were collected using differential interference contrast (DIC). Digital image recording and image analysis were performed with the Axiovision 3.1 software (Zeiss).

Flow Cytometric Analysis of Cellular Polyplex Association

Cells were seeded in 12 well plates (TPP) at a density of 75,000 cells per well 24 h prior to transfection. Polyplexes containing Cy5-labeled DNA (20% Cy5-DNA) were added to the cells at a DNA concentration of 3 μg per well (150 μl polyplex solution) in 1 ml of fresh culture medium with FCS and incubated at 37°C for indicated periods of time. At certain time points cells were washed twice with PBS, harvested by treatment with trypsin/EDTA and stored on ice until further analysis. Cell association of polyplexes was assayed using a Cyan MLE flow cytometer (Dako, Copenhagen, Denmark). The fluorophore Cy5 was excited at 635 nm and emission was detected at 665/20 nm. Data acquisition was performed in linear mode and data were analyzed in logarithmic mode. To discriminate between viable and dead cells and to exclude doublets, cells were appropriately gated

Table II. Size and Zeta Potential of PAHy–CPTA18 Polyplexes at a Charge Ratio of 7.6 Polymer/DNA (20:1 *w/w* ratio)

buffer	DNA (μg/ml)	size (nm)	PI	Zeta potential (mV)
HBG	20	358	0.04	n.d.
HBG	200	408	0.07	5.8±1.6
HBS1/2	20	1350	0.03	2.8±1.6

PI Polydispersity index

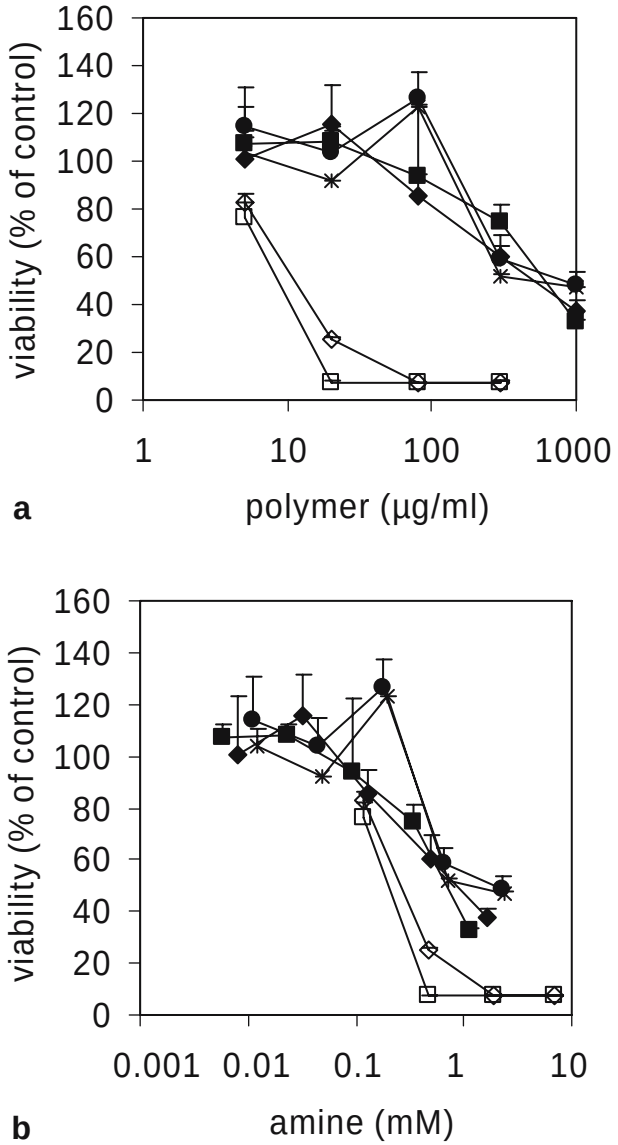

Fig. 2. Cytotoxicity of polymers *in vitro*. 5,000 Neuro2A cells were seeded in 96-well plates 24 h prior to incubation with polymers. The indicated amount of polymer in 100 μl of serum containing medium was incubated with cells for 48 h, thereafter MTT activity was measured as described in MATERIALS AND METHODS. Relative values compared to untreated control are presented. MTT activity is plotted *vs.* polymer/DNA *w/w* ratio (**a**) or molar ratios of amine concentration (for PEI: primary, secondary and tertiary, for PAHy–CPTA quaternary) to phosphate in pDNA (**b**). Mean values from triplicates±standard deviation are shown. *Open diamond* LPEI; *open square* BPEI; *filled square* PAHy–CPTA18; *filled diamond* PAHy–CPTA33; *filled circle* PAHy–CPTA48; *cross* PAHy–CPTA58

by forward/side scatter and pulse width; 2×10^4 gated events per sample were collected. Experiments were performed at least in triplicates.

In Vivo Application, Systemic

A/J mice (10–12 weeks old, female, 20–23 g) were housed in individually vented cages under specified pathogen free conditions with a 12 h day/night cycle; food and water were provided *ad libitum*. Animals received intravenous injections of polymer solution or polyplexes via the tail vein. PAHy–CPTA18 polymer was applied at a concentration of 4 mg/ml, LPEI at 0.2 mg/ml in sterile HBG. 48 hours after application animals were sacrificed and perfusion fixed with formalin (4% paraformaldehyde in PBS buffer). Livers were explanted and embedded in paraffin. Five-micrometer thick sections of mice liver were stained with hematoxylin and eosin for histological analysis. Polyplexes were generated with an N/P ratio of 6 (LPEI) or a charge ratio of 7.6 (*w/w* 20) PAHy–CPTA18/DNA) with a final plasmid DNA concentration of 200 μg/ml. Control animals received 250 μl HBG alone or were not treated at all. In case of polyplex applications, body weight of animals was measured before application, 24 and 48 h after injection.

In Vivo Application, Intratumoral

A/J mice (6–7 weeks old, female) were injected subcutaneously with 10^6 Neuro2A cells in 100 μl sterile PBS per site as described (11), one each on the right and the left flank of the animal. When tumors reached a diameter of approx. 10 mm, mice were anesthetized with 2% isofluoran in oxygen and 100 μl of pDNA or polyplex solution (final DNA concentration 200 μg/ml in HBG) were applied by

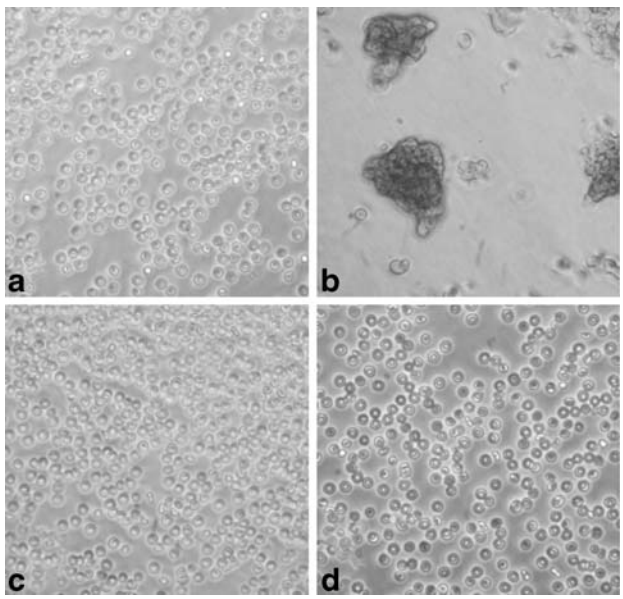

Fig. 3. Erythrocyte aggregation induced by polymers. Erythrocytes were incubated with polymer solution as described in "MATERIALS AND METHODS." for 20 min at room temperature. The suspension was viewed after further dilution of the suspension (1:10 in HBG buffer). **a** Control; **b** 10 μg LPEI; **c** 100 μg PAHy–CPTA48; **d** 100 μg PAHy–CPTA58

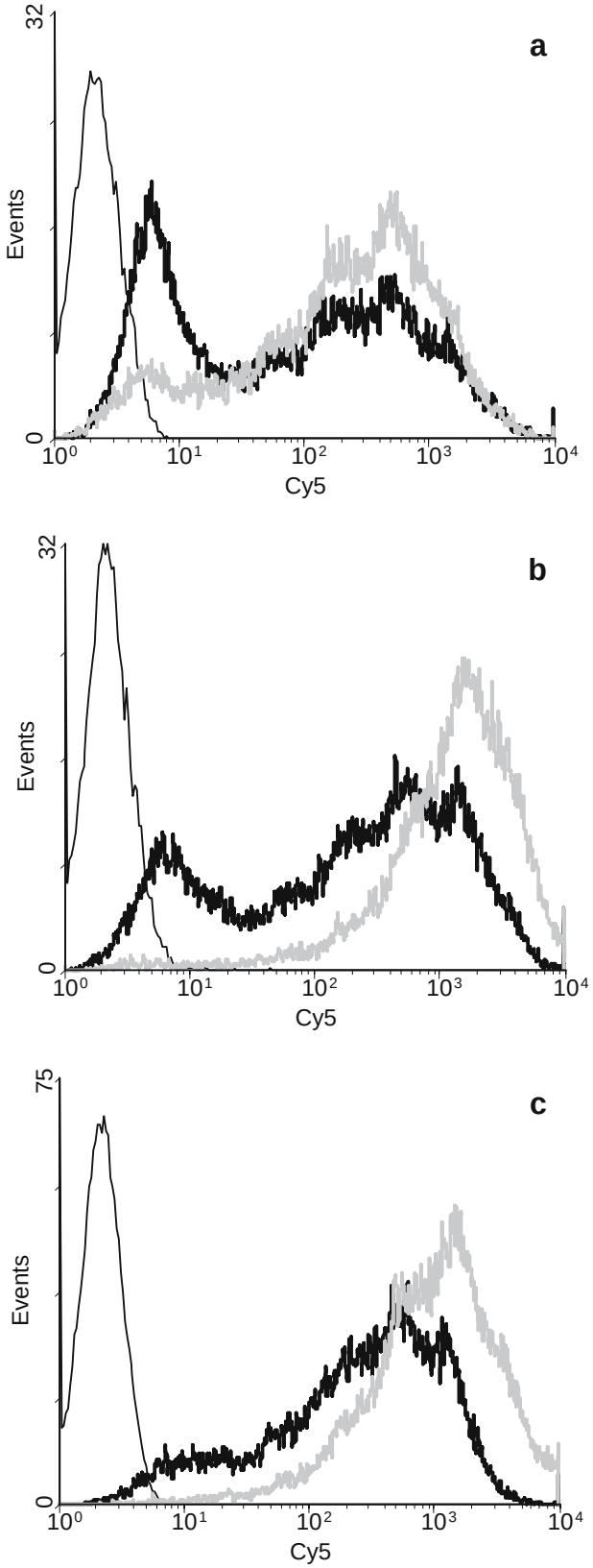

Fig. 4. Cellular association of polyplexes with Neuro2A cells. Cells were incubated with Cy5-labelled polyplexes for 4 (**a**), 8 (**b**) or 24 h (**c**), harvested and analyzed as described in "MATERIALS AND METHODS." *Thin line* control cells; *black line* LPEI/DNA N/P 6; *grey line* PAHy–CPTA18/DNA charge ratio 7.6. Experiments were carried out at least in triplicates, representative curves are shown

intratumoral application using a 25 G needle. 48 h after application animals were sacrificed, tumors resected and stored at −80°C. Tumors were homogenized in cell lysis buffer (Promega, Mannheim, Germany) using an IKA-Ultra-Turrax and luciferase activity of the lysates was determined as described above. Luciferase background (approximately 200 RLU) was subtracted from each value and transfection efficiency is expressed as RLU per 100 mg tumor tissue (mean±standard deviation, n=4–6).

All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

STATISTICAL ANALYSIS

Where indicated, one-way analysis of variance (ANOVA) with subsequent Duncan test was employed.

RESULTS AND DISCUSSION

The synthesis of α,β-polyaspartylhydrazide-carboxypropyltrimethylammonium chloride copolymers (PAHy–CPTA) used in this study have been performed as previously described (8). Briefly, the covalent linkage of carboxypropyltrimethylammonium chloride (CPTACl) to hydrazide group of PAHy was performed in water solution at pH 4.75 by using *N′*-ethyl-*N″*-dimethylaminopropylcarbodiimide (EDC) as coupling agent. The degree of derivatization (DD%), indicated as percentage of positive charged groups in comparison with repeating units of PAHy, was calculated by ^{1}H-NMR as reported elsewhere (8). Four copolymers differing in DD % were obtained by using increasing EDC amounts, expressed as molar ratio of moles EDC and moles of CPTACl (Y=0.25, 0.5, 1.0 or 1.5); copolymers were purified by exhaustive dialysis and obtained with a yield ranging from 90 to 97 *w*/*w* % based on starting PAHy. Main molecular parameters of PAHy–CPTA copolymers are reported in Table I. As expected as DD % increases, the molecular weight/positive charge ratio decreases. Moreover Table I shows that the molecular weights of PAHy–CPTA copolymers are slightly lower than that of parent PAHy indicating that during reaction between CPTA and PAHy, in the presence of EDC, a limited degradation process along the PAHy polymeric backbone occurs.

PAHy–CPTA copolymers at DD % of 18, 33, 48 and 58 were then used for subsequent studies of polyplex formation and characterization.

Biophysical Properties of DNA/PAHy–CPTA Polyplexes

The extent of quenching/exclusion of ethidium bromide (EtBr) bound to DNA by polycations is a measure for the degree of DNA compaction obtained (12). Stepwise addition of LPEI to EtBr labeled DNA resulted in rapid decrease of the fluorescence signal measured (Fig. 1). Already at a polymer/DNA ratio of 0.5 (*w*/*w*) (corresponding to an N/P ratio of 3.8) 90% of the initial signal was quenched. In sharp contrast, all PAHy–CPTA derivates were able to decrease the signal only to approx. 50% at polymer/DNA weight ratio 40. This also indicates that considerable amounts of PAHy–CPTA are free in solution at *w*/*w* ratios>5. In the case of PEI based polyplexes, at N/P ratio of 6 (PEI/DNA 0.78/1 *w*/*w*)

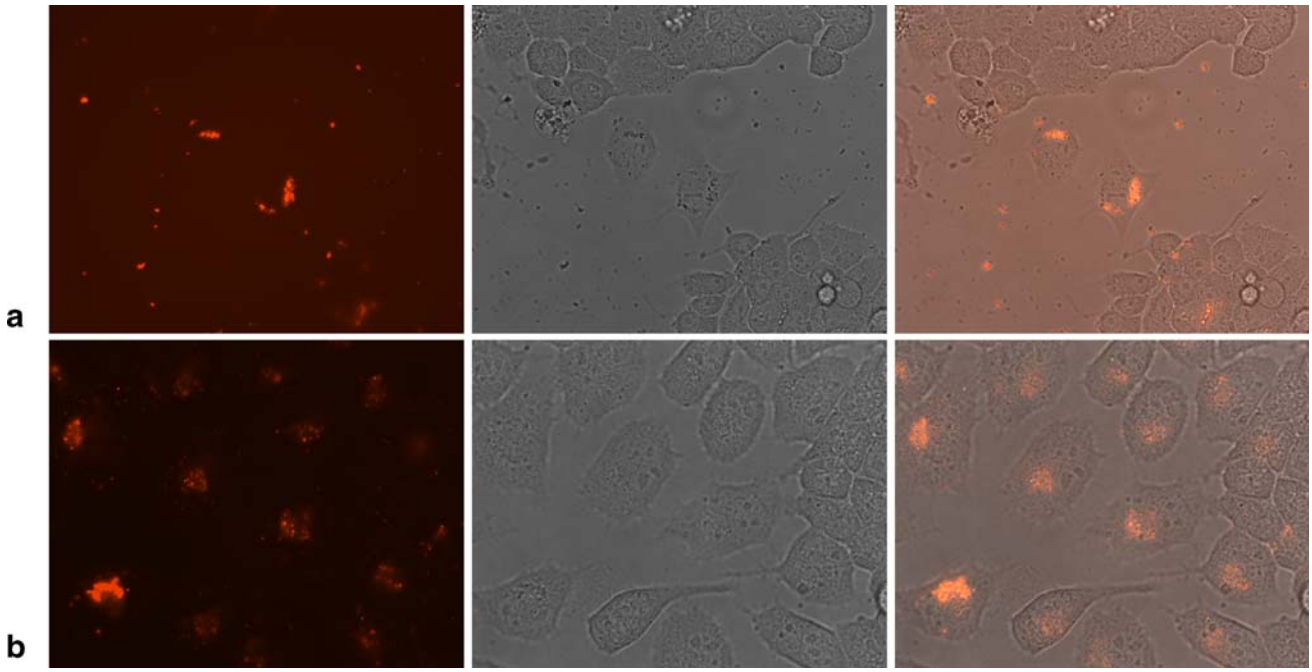

Fig. 5. Intracellular distribution of polyplexes. Neuro2A cells were incubated with Cy3-labelled polyplexes for 24 h. **a** LPEI polyplexes *N*/*P* 6 in HBS1/2; **b** PAHy–CPTA58 polyplexes at *N*/*P* ratio 17 (corresponding to *w*/*w* polymer/DNA ratio of 20/1) in HBS1/2. *Left panel* fluorescence, *middle panel* transmitted light (DIC), *right panel* overlay

40–60% of PEI appears in free, non-DNA bound form [see (10)]. This correlates well with the agarose retardation assay, were complete retardation of pDNA with BPEI and LPEI is observed at N/P ratios of 2–3. In the case of PAHy–CPTA, complete retardation of DNA is observed for PAHy–CPTA 48 for *w*/*w* ratios between 2 and 3 [corresponding to *N*/*P* ratios of 1.5:2.2; see (8)]. From these data we conclude that e.g. at a *w*/*w* ratio of 20/1 PAHy–CPTA 48/pDNA, 80–90% of the polymer is free in solution. Although efficient retardation of DNA can be achieved, the polyplexes formed appeared to have a rather loose structure. In terms of protection by nucleases this condensations seems sufficient (8).

Here we show particle size and zeta potential of PAHy–CPTA18 polyplexes with plasmid DNA either measured in a low ionic buffer (HBG) or in the presence of salt (HBS1/2) (Table II). Similar to PEI polyplexes (13), PAHy–CPTA polyplexes were aggregated in HBS1/2 and medium sized (300–400 nm) in HBG. Whereas polyplexes in HBS1/2 further aggregated with time, polyplexes generated in HBG remained stable for at least 2 h. For all PAHy–CPTA polyplexes zeta potential was below 10 mV, even when using a 100/1 polymer/DNA *w*/*w* ratio (corresponding to *N*/*P* ratio of 38, 56, 76 and 85 respectively for PAHy–CPTA 18, 33, 48 and 58%; data not shown).

Apparently the capacity of PAHy–CPTA being bound to plasmid DNA is limited and the density of positive charge is much lower compared to PEI, where every third atom in the polymer backbone is a protonable nitrogen.

Cytotoxicity and Induction of Erythrocyte Aggregation

Lack of toxicity is a key issue for a further *in vivo* application of polycations. Neuro2A murine neuroblastoma cells were incubated for 48 h with increasing concentrations of polymer in serum containing medium (Fig. 2). As expected, LPEI was highly toxic already at low concentrations: >90% of cells were killed at 20 μg/ml or above. Similar to results obtained with PAHy–GTA (7), PAHy–CPTA did not result in any cytotoxicity up to 80 μg/ml polymer, and at 1 mg/ml only 50% toxicity was observed. When calculating the concentration of polymer used based on amine (for PEI primary, secondary and tertiary amines, for PAHy–CPTA derivates quaternary amines), a rather similar trend was observed: whereas with BPEI and LPEI 50% toxicity was observed between 0.1 and 0.5 mM amine, PAHY–CPTA induced the same toxicity between 0.7 and 2 mM amine. There was no significant difference within the four PAHy–CPTA derivatives indicating that within this range the degree on derivatization of PAHy does not correlate with cytotoxicity.

Polycationic molecules have the tendency to aggregate erythrocytes due to electrostatic interaction with negatively charged sialic acid residues, and aggregation behavior *in vitro* correlates with toxicity *in vivo* (14). This aggregation behavior can lead to acute toxicities after systemic application *in vivo* due to lung embolism (11,13,15). As already described by other groups and us, LPEI rapidly aggregated erythrocytes already at low polymer concentrations (Fig. 3). In contrast, even the PAHy–CPTA derivate with the highest degree of derivatization (58%) did not induce any aggregation, also when increasing the polymer concentration to 1 mg/ml.

Cellular Interaction of PAHy–CPTA Polyplexes

Binding of polymeric gene carrier to the cell surface occurs by interaction of the usually positively charged polyplex with cellular heparane sulfates (16,17). We have carried out binding studies by flow cytometry and epifluorescence microscopy incubating Neuro2A with fluorescently labeled polyplexes. Prior to condensation DNA was covalently modified with either Cy5 (flow cytometry) or Cy3 dye

(fluorescence microscopy). This type of labeling allows reliable tracking of plasmid DNA, as the covalent type of labeling prevents dissociation of the dye. Flow cytometry revealed rapid cellular association of both LPEI polyplexes and PAHy–CPTA polyplexes already after four hours (Fig. 4). At this time point 60% of cells transfected with LPEI polyplexes showed high fluorescence, whereas with PAHy–CPTA polyplexes >80% of cells were highly fluorescent. After total eight hours of incubation maximal cellular association was obtained with PAHy–CPTA polyplexes, whereas cell binding for LPEI polyplexes was further increased after 24 hours. This indicates that cell binding of PAHy–CPTA/DNA is even faster and more pronounced then with LPEI/DNA, and the amount of cellular associated plasmid increases with time. Although PAHy–CPTA polyplexes exhibit a lower surface charge compared to LPEI polyplexes (+5 mV *vs*. +30 mV), this fast cellular binding with cells occurs. Microscopy studies have been carried out after 24 h of incubation with polyplexes. Differences in the intracellular distribution patter for the polyplexes can be observed (Fig. 5). LPEI polyplexes form large aggregates on and in the cells and also on the plastic surface being visible also in transmitted light. Similar observations have been already described in the literature (13). Besides other effects, like endosomal buffering, the aggregation of LPEI polyplexes is responsible for its very high transfection efficiency *in vitro*. Comparing the aggregation behavior of BPEI and LPEI, Wightman *et al.* (13) could show that LPEI polyplexes have a much higher tendency to aggregation compared to BPEI polyplexes leading to very high levels of transgene expression *in vitro*. Nevertheless, for *in vivo* delivery such massive aggregation will prevent polyplexes to reach distant organs after systemic application and result in the entrapment in the lung. DNA delivered by PAHy–CPTA poly-

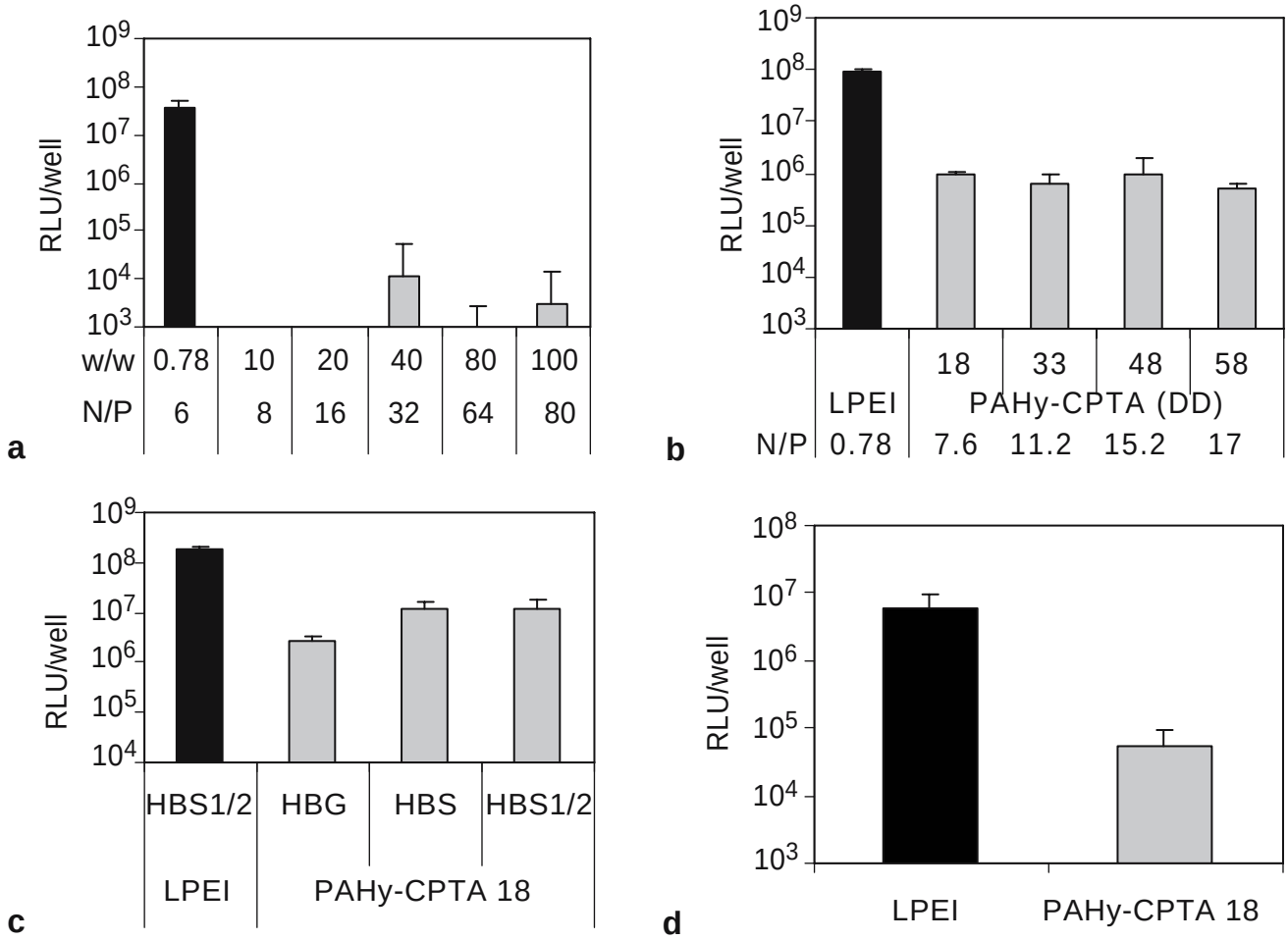

Fig. 6. *In vitro* transfection of tumor cells. Cells were transfected in 96-well plates in 100 µl medium containing FCS either with pCMVLuc/LPEI polyplexes (*N*/*P* 6 in HBS1/2, *black bars*) or pCMVLuc/PAHy–CPTA polyplexes (as indicated, *grey bars*). **a** Neuro2A cells were transfected with 200 ng DNA per well for 4 h. Thereafter medium was replaced by 200 µl fresh medium and luciferase assay carried out after 24 h. pCMVLuc/PAHy–CPTA58 polyplexes were generated in HBS1/2 with different *w*/*w* (*N*/*P*) ratios as indicated in the figure. **b** Neuro2A cells were transfected with 500 ng pCMVLuc per well for 48 h and luciferase assay carried out thereafter. pCMVLuc/PAHy–CPTA polyplexes were generated in HBS1/2 with polymer/DNA *w*/*w* ratio of 20:1 corresponding to a *N*/*P* ratio of 7.6, 11.2, 15.2 and 16 for PAHy–CPTA 18, 33, 48 and 58% respectively. PAHy–CPTA copolymers with different degree of derivatization were used as indicated. **c** Neuro2A cells were transfected as in B. pCMVLuc/PAHy–CPTA18 polyplexes were generated in the indicated buffer with *N*/*P* ratios of 7.6. **d** HuH7 cells were transfected as in (**b**). pCMVLuc/PAHy–CPTA18 polyplexes were generated in HBS1/2 with *N*/*P* ratio of 7.6. Mean values of triplicates±standard deviation are shown

plexes was found in rather small compartments inside the cell and no larger aggregates were seen in transmitted light. Apparently there was efficient internalization of DNA achieved with PAHy–CPTA polyplexes.

In Vitro Transfections

Having shown the low toxicity and efficient internalization of PAHy–CPTA polyplexes, transfection studies using luciferase were carried out on two different tumor cell lines.

Transfection of the murine neuroblastoma cell line Neuro2A revealed that under standard transfection conditions usually used for LPEI polyplexes (4 h incubation period of complexes with cells, subsequent removal of polyplexes and assay for reporter gene expression 24 h thereafter) only very low levels of luciferase expression can be achieved with PAHy–CPTA polyplexes (Fig. 6a). A considerable increase in reporter gene expression was achieved when keeping the polyplexes on the cells for up to 48 h (B). A significantly increase in reporter gene expression was observed using 500 ng or compared to 200 ng (data not shown) DNA per well. Transfection with 500 ng resulted in slightly higher expression values compared to 200 ng. In separate experiments, different polymer/DNA charge ratios were tested (data not shown). For example between a 3.8 and 38 polymer/DNA charge ratio for PAHy–CPTA 18 or 8.5 and 85 polymer/DNA charge ratio for PAHy–CPTA 58 there was no significant difference observed. Although cellular binding of polyplexes was very efficient already after four hours of incubation (Fig. 4), this was obviously not enough to obtain efficient transgene

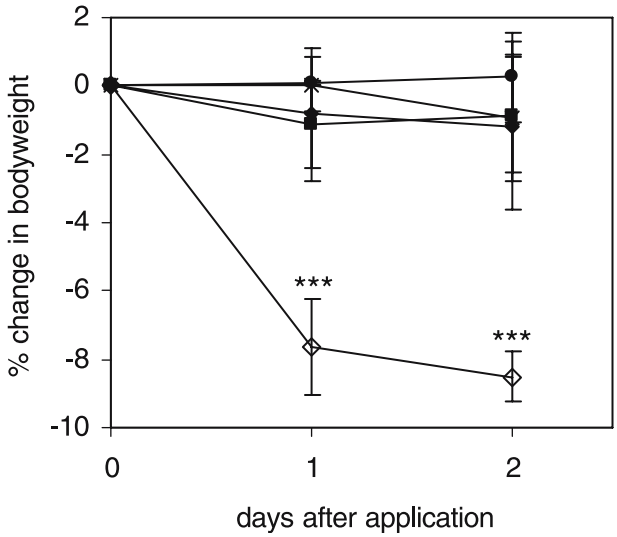

Fig. 8. Systemic application of polyplexes. Polyplexes were injected at a dose of 2.5 mg/ml DNA into the tail vein of A/J mice. Bodyweight of animals was monitored prior to injection, 1 and 2 days thereafter and the relative weight loss calculated. Mean values obtained from two to eight animals±standard deviation are shown. ***$p<0.001$, LPEI compared to all other groups (ANOVA, Duncan test). *Open diamond* LPEI; *filled square* PAHy–CPTA18; *filled diamond* PAHy–CPTA33; *filled circle* PAHy–CPTA48; *cross* PAHy–CPTA58

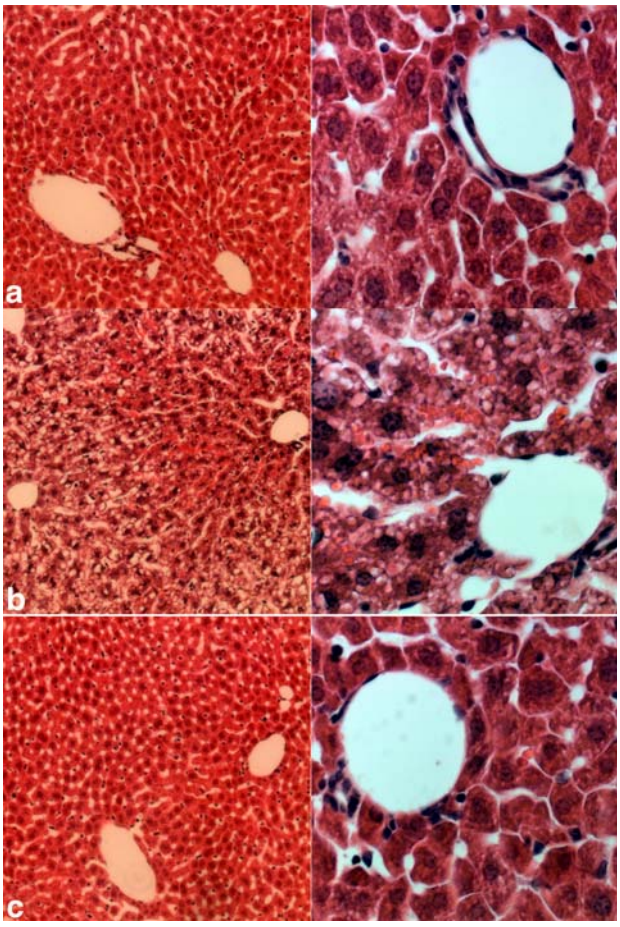

Fig. 7. Liver histology after polymer application. Polymers were injected as described in materials and methods at a dose of 2.5 mg/kg for LPEI and 50 mg/kg for PAHy–CPTA18. **a** HBG only, **b** LPEI, **c** PAHy–CPTA18; *left panel* final magnification 50-fold; *right panel* final magnification 580-fold

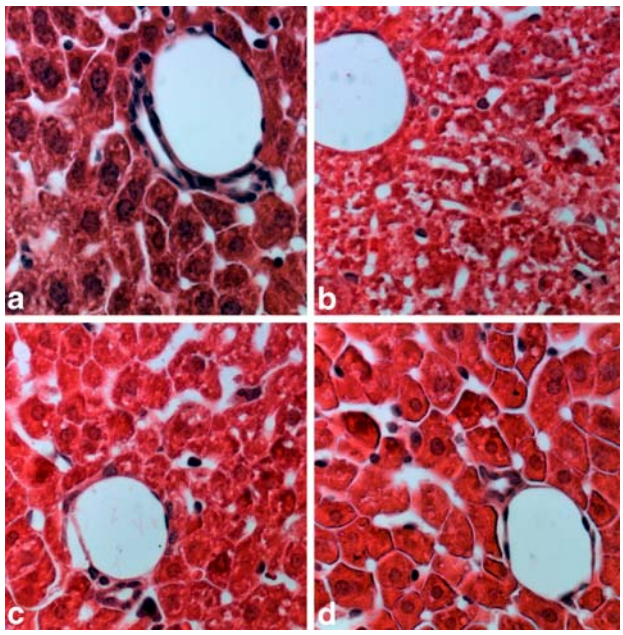

Fig. 9. Liver histology after polyplex application. Mice were injected with HBG (**a**), LPEI/DNA polyplexes (**b**), PAHy–CPTA58/DNA polyplexes (**c**) or PAHy–CPTA58 (**d**) and sacrificed 48 h after injection; final magnification 580-fold

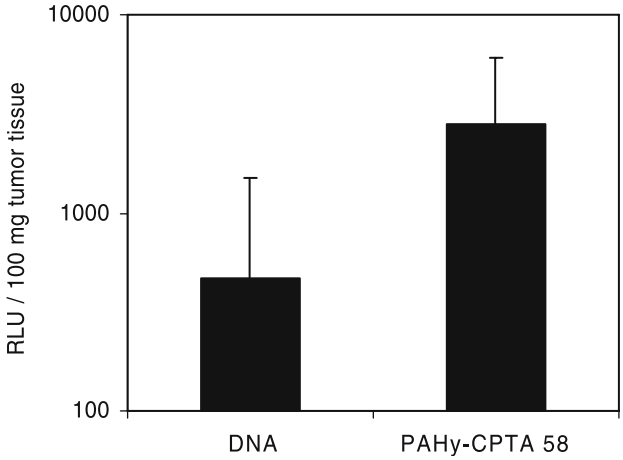

Fig. 10. Transgene expression after intratumoral application. Mice were injected intratumorally with 100 μl of naked DNA or PAHy–CPTA polyplexes (*w*/*w* ratio 20:1 PAHy–CPTA 58/DNA, *N*/*P* ratio 16:1) containing 20 μg of pDNA. Luciferase expression was quantified 48 h after injection as described in "MATERIALS AND METHODS." Mean values from four to six tumors+stddev are shown

expression when exchanging the transfection medium after four hours. The total amount of cell bound DNA obtained using PAHy–CPTA polyplexes is already higher then the one obtained with LPEI polyplexes. So we conclude from the flow cytometry experiments but also from the transfection results in Fig. 6b (increased incubation time) that further internalization of PAHy–CPTA is necessary for efficient transgene expression. We have recently observed that free LPEI enhances transfection of LPEI polyplexes and colocalizes within intracellular vesicles leading to improved endosomal release (10). It is conceivable that free PAHy–CPTA can cause a similar effect, but within an extended period of time. In separate experiments we have also investigated the influence of the endosomolytic drug chloroquine on PAHy–CPTA polyplex transfection (data not shown). A ten-fold increase in luciferase expression was achieved with 25 μM chloroquine present during 48 h of transfection. Further increasing the drug concentration led to decreased transgene expression due to toxic side effects of the drug. The buffer composition strongly influences the aggregation behavior of polyplexes with PEI (13,18). It was recently shown that HEPES buffer containing 75 mM salt was optimal for generation of LPEI polyplexes with a size of one micrometer resulting in maximal reporter gene expression on different tumor cell lines including Neuro2A (13). Although also PAHy–CPTA polyplexes aggregated in the presence of salt (see Table II), this had less influence on transfection efficiency (Fig. 6c). Nevertheless, using HBS1/2 or HBS as a complexation buffer a six-fold increase in luciferase expression was found compared to PAHy–CPTA polyplexes generated in HBG. Besides Neuro2A cells also the human hepatoma cell line HuH7 was transfected under similar conditions (Fig. 6d). Again, with LPEI polyplexes a high transfection level was found, transfection efficiency with PAHy–CPTA polyplexes was approx. 1% of LPEI polyplexes.

Transfection activity of PAHy–CPTA polyplexes is in the best case ten fold lower compared to optimized LPEI polyplexes; but it has to be mentioned that in comparison with PEI polyplexes based on branched PEI transfection levels are similar (A. Kotha, M. Ogris, unpublished results). All transfections with PEI and PAHy–CPTA based polyplexes were carried out at final polymer concentration showing less then 50% toxicity when applied without DNA (as shown in Fig. 2).

In Vivo Application

From our previous observations we knew that at elevated PAHy–CPTA/DNA ratios (20/1 *w*/*w*), optimal protection against enzymatic DNA degradation can be achieved *in vitro* (8). Hence, we have chosen the 20/1 *w*/*w* ratio (corresponding to an *N*/*P* ratio of 7.5 (PAHy–CPTA 18), 10.7 (PAHy–CPTA 33), 14.7 (PAHy–CPTA 48) and 16 (PAHy–CPTA 58) for the systemic application of polyplexes *in vivo*.

First, polymers alone (2.5 mg/kg LPEI or 50 mg/kg PAHy–CPTA18) were injected intravenously into A/J mice and the liver examined for histopathological changes 48 h after injection (Fig. 7). Livers of animals receiving LPEI showed signs of microvesicular fatty liver in the periphery, which is also described after acute and chronic intoxication. Although PAHy–CPTA polymers were injected with a 20-fold higher dose (PAHy–CPTA18 in Fig. 7c, PAHy–CPTA58 in Fig. 9d), no histopathological changes were observed.

After intravenous application of LPEI polyplexes mice showed slight signs of shock, like ruffled fur and reduced activity. Significant toxicity was observed after 24 h and 48 h in terms of weight loss (Fig. 8). In sharp contrast, no weight loss was observed for PAHy–CPTA polyplexes applied at a dose of 50 mg/kg PAHy–CPTA (Fig. 8). Two days after polyplex application, the liver was also examined for histopathological changes (Fig. 9). LPEI polyplexes caused significant liver damage (Fig. 9b), although lo to a lesser extent compared to LPEI alone. Similar observations were also made by Chollet et al (15), where damage of lung and liver tissue was found. Again, with PAHy–CPTA58 (9C) or PAHy–CPTA58 polyplexes (9D) no histopathological changes were observed.

No significant levels of transgene expression were observed when injecting PAHy–CPTA based polyplexes intravenously into mice (M. Ogris, unpublished observations). Hence, we carried out intratumoral injections in A/J mice bearing subcutaneous implanted Neuro2A tumors. A measurable, but low transgene expression was found for PAHy–CPTA 58 polyplexes (Fig. 10), which was approx. five-fold higher compared to naked pDNA. Currently we are evaluating different cationic side chains for PAHy modification with some of them being efficient in gene delivery both *in vitro* and *in vivo* (manuscript in preparation).

CONCLUSIONS

In summary it can be said that PAHy–CPTA has an excellent toxicity profile both *in vitro* and *in vivo* being almost non toxic even at elevated polymer doses. Polyplexes with plasmid DNA are efficiently internalized by cells and lead to considerable levels of transgene expression. These properties make PAHy–CPTA a promising candidate for systemic gene delivery *in vivo*. Since the transfection efficiency *in vitro* and *in vivo* is still improvable, we are

now evaluating other polycationic gene delivery vectors based on PAHy for tumor gene delivery.

ACKNOWLEDGMENTS

This work was in part funded by the Deutsche Forschungsgemeinschaft, SPP1230, SFB 486 'Nanoman', excellence cluster 'NIM' and by the European Community FP6 network project 'GIANT'. This work was also partially funded by MIUR–ITALY grants.

REFERENCES

1. M. Thomas and A. M. Klibanov. Non-viral gene therapy: polycation-mediated DNA delivery. *Appl. Microbiol. Biotechnol.* **62**:27–34 (2003).
2. M. Ogris and E. Wagner. Targeting tumors with non-viral gene delivery systems. *Drug Discov. Today* **7**:479–485 (2002).
3. D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, and T. Kissel. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **24**:1121–1131 (2003).
4. K. A. Howard, P. R. Dash, L. Read, K. Ward, L. M. Tomkins, O. Nazarova, K. Ulbrich, and L. W. Seymour. Influence of hydrophilicity of cationic polymers on the biophysical properties of polyelectrolyte complexes formed by self-assembly with DNA. *Biochim. Biophys. Acta* **1475**:245–255 (2000).
5. G. Giammona, B. Carlisi, G. Cavallaro, G. Pitarresi, and S. Spampinato. A new water-soluble synthetic-polymer, alpha,-beta-polyasparthydrazide, as potential plasma expander and drug carrier. *J. Controlled Rel* **29**:63–72 (1994).
6. G. Giammona, V. Tomarchio, G. Pitarresi, and G. Cavallaro. Glycidyl methacrylate derivatization of α,β-poly(*N*-hydroxyethyl)-DL-aspartamide and α,β-polyasparthydrazide. *Polymer* **38**:3315–3323 (1997).
7. E. Pedone, G. Cavallaro, S. C. Richardson, R. Duncan, and G. Giammona. α,β-poly(asparthylhydrazide)-glycidyltrimethylammonium chloride copolymers (PAHy–GTA): novel polymers with potential for DNA delivery. *J. Control Release* **77**:139–153 (2001).
8. G. Cavallaro, F. S. Palumbo, M. Licciardi, and G. Giammona. Novel cationic copolymers of a polyasparthylhydrazide: synthesis and characterization. *Drug Deliv.* **12**:377–384 (2005).
9. J. Kloeckner, E. Wagner, and M. Ogris. Degradable gene carriers based on oligomerized polyamines. *Eur. J. Pharm. Sci.* **29**:414–425 (2006).
10. S. Boeckle, K.von Gersdorff, S.van der Piepen, C. Culmsee, E. Wagner, and M. Ogris. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J Gene Med* **6**:1102–1111 (2004).
11. 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).
12. A. L. Parker, D. Oupicky, P. R. Dash, and L. W. Seymour. Methodologies for monitoring nanoparticle formation by self-assembly of DNA with poly(*l*-lysine). *Anal. Biochem.* **302**:75–80 (2002).
13. 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).
14. E. Moreau, M. Domurado, P. Chapon, M. Vert, and D. Domurad. Biocompatibility of polycations: *in vitro* agglutination and lysis of red blood cells and *in vivo* toxicity. *J. Drug Target* **10**:161–173 (2002).
15. 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).
16. I. Kopatz, J. S. Remy, and J. P. Behr. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J Gene Med* **6**:769–776 (2004).
17. K.A. Mislick and J.D. Baldeschwieler. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. U. S. A* **93**:12349–12354 (1996).
18. 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).