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Effect of cationic carriers on the pharmacokinetics and tumor localization of nucleic acids after intravenous administration

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Abstract

Nucleic acid based therapeutics are currently being studied for their application in cancer therapy. In this study, the effect of different cationic delivery systems on the circulation kinetics, tumor localization, and tissue distribution of short interfering RNA (siRNA) and plasmid DNA (pDNA) was examined, after intravenous administration in mice bearing a s.c. Neuro 2A tumor. Nanosized particles were formed upon complexation of siRNA with the cationic liposome formulation DOTAP/DOPE and the targeted, cationic polymer RGD-PEG-PEI. Both the circulation kinetics and the overall tumor localization of the siRNA complexes were similar to non-complexed siRNA. Importantly, the different carriers changed the intratumoral distribution of siRNA within the tumor. pDNA was effectively condensed with linear polyethylenimine (PEI), PEGylated linear PEI (PEG-PEI) or poly(2-dimethylamino ethylamino)phosphazene. Only PEG-PEI was able to improve the pDNA circulation kinetics. All pDNA complexes yielded similar pDNA tumor localization (1% of the injected dose, 60 min after administration). We conclude that the level of nucleic acid tumor localization is independent on the type of formulation used in this study. Therefore, the value of carrier systems for the intravenous delivery of nucleic acids cannot be solely attributed to benefits relevant during the transport towards the tumor. Rather, the benefits are arising from carrier-induced changes in the intratumoral fate of the nucleic acids.
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Keywords: siRNA complexes; pDNA complexes; Polyplexes; Tumor localization; Circulation kinetics; Intravenous administration

1. Introduction

Nucleic acid based therapeutics are currently being studied for their application in cancer therapy as an alternative to chemotherapeutics and protein drugs. Plasmid DNA (pDNA) and short interfering RNA (siRNA) show high promise (Schiffelers et al., 2004b; Dong and Woraratanadharm, 2005; Lage, 2005). However, as a consequence of the physicochemical nature of nucleic acids, their cellular uptake is limited and intracellular processing inefficient. Complexation of the nucleic acids with cationic carrier systems can strongly improve their cellular interaction (Merdan et al., 2002; Pack et al., 2005; Behlke, 2006). The resulting, positively charged nanoparticles have demonstrated improved cellular adhesion and a more efficient intracellular trafficking.

In addition, upon intravenous administration, nucleic acids encounter difficulties in reaching the tumor site. They are degraded within the bloodstream by nucleases and are rapidly cleared by the non-parenchymal cells of the liver. Furthermore, the siRNA or the pDNA fragments are subjected to efficient glomerular kidney filtration (Takakura et al., 2002; Van De Water et al., 2006). As a consequence, nucleic acids show poor pharmacokinetics after intravenous administration together with a low level of accumulation at the distant tumor site. Again, complexation of nucleic acids with cationic carriers has proven to be of great value. For example, nucleic acids complexed with cationic carriers have demonstrated resistance against nuclease digestion (Garcia-Chaumont et al., 2000; Pack et al., 2005). Generally, carrier systems are considered indispensable for efficient nucleic acid cancer therapy after intravenous administration.

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The organ gene expression profiles of the nucleic acid complexes are strongly affected by both the nature of the carrier molecules, as well as the extent of surface modification, i.e. the possible presence of a shielding or targeting moiety. Therefore, by carefully choosing the composition of a particular carrier system, one may tailor its properties for the desired application. So far, the intravenous administration of different cationic lipid or polymer complexes has resulted in significant tumor gene expression or silencing in different animal models (Templeton, 2002; El-Aneed, 2004; Wagner, 2004; Behlke, 2006).

Up to now, only limited attention has been paid to address the effect of complexation on the pharmacokinetics and tumor localization of nucleic acids. In this study, we examined the blood, tumor and tissue levels of different, ^{35}S-labeled, siRNA and pDNA complexes after intravenous injection in mice. The investigated carrier systems were chosen for their promise in cancer gene therapy, as assessed by reporter gene assays and tumor growth studies (Templeton et al., 1997; Wolschek et al., 2002; Schiffelers et al., 2004a; de Wolf et al., 2005; Lungwitz et al., 2005).

2. Materials and methods

2.1. Materials

Small interference RNA (siRNA) against luciferase and FITC-labeled siRNA were obtained from Qiagen (Venlo, The Netherlands). Endotoxin-free plasmid encoding for firefly luciferase, pcDNA3Luc, was obtained from PlasmidFactory (Bielefeld, Germany). The lipids 1,2-dioleyl-3-trimethylammonium-propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar lipids (Alabaster, USA) and Lipoid (Ludwigshafen, Germany), respectively.

Branched polyethylenimine (M_w 25 kDa) was obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). RGD-PEG-PEI, branched PEI (M_w 25 kDa) grafted with distally modified RGD-$PEG_{3.4\,kDa}$, was synthesized as described previously (Schiffelers et al., 2004a). Both linear polyethylenimine (PEI, M_w 22 kDa) and PEG-PEI, linear $PEI_{22\,kDa}$ to which polyethylene glycol (PEG) (M_w 20 kDa) is grafted (in a molar ratio of 1–1.8), were a generous gift from Prof. Dr. E. Wagner (Ludwig-Maximilians-Universität München) (Wolschek et al., 2002; Kursa et al., 2003). Poly(2-dimethylamino ethylamino)phosphazene (p(DMAEA)-ppz, M_w 49 kDa) was synthesized as described previously (Luten et al., 2003). Cytidine 5′-[alpha-^{35}S]thiotriphosphate (α-^{35}S CTP), deoxycytidine 5′-[alpha-35S]thiotriphosphate (α-^{35}S dCTP), and the Nick Translation Kit were purchased from GE Healthcare Europe (Roosendaal, The Netherlands). Solvable solubilizer and Ultima Gold scintillation liquid were supplied by Perkin-Elmer BV (Groningen, The Netherlands).

2.2. Methods

2.2.1. Preparation of ^{35}S-labeled siRNA and ^{35}S-labeled pDNA

Double-stranded, radiolabeled siRNA (aacttacgctgagtacttcga and aatcgaagtactcagcgtaag) was constructed and purified using the silencer siRNA construction kit (Ambion Europe, Cambridgeshire, UK) according to the instruction manual. Two 29-mer DNA oligonucleotides with 21 nt encoding the siRNA and 8 nt complementary to the T7 Promoter Primer served as siRNA transcription templates (Isogen Lifescience, Maarssen, The Netherlands). α-^{35}S CTP was incorporated in the NTP transcription mixture. The integrity of the hybridized construct was confirmed using agarose gel electrophoresis and UV measurements at 260 and 280 nm.

Plasmid DNA (pDNA), pcDNA3Luc, was labeled with α-^{35}S dCTP by Nick Translation according to the instruction manual. The product was purified using a G-50 Sephadex column.

Before complex formation, unlabeled nucleic acid solutions were spiked with the radiolabeled counterparts.

2.2.2. Preparation of the complexes

All complexes with siRNA or pDNA were prepared by vortexing equal volumes of nucleic acid solutions with the different carrier system solutions. All complexes were incubated for 30 min at room temperature, prior to injection. Different molar excesses of ionizable nitrogen (cationic charge carrier system) to phosphate (nucleic acids) (N/P ratio) were applied for the different complexes, similar to the ones applied in previous publications (Wightman et al., 2001; de Wolf et al., 2005; Schiffelers et al., 2005).

DOTAP/DOPE liposomes were prepared by dissolving an equimolar amount of DOTAP and DOPE in chloroform and methanol. The solution was evaporated under reduced pressure and the lipid film was subsequently hydrated in Hepes 10 mM, pH 7.4 (final lipid concentration was 10 μM). The resulting liposome dispersion was extruded several times through two stacked polycarbonate membranes until the desired diameter was obtained (100–120 nm).

Formulations of siRNA were prepared in a 10 mM Hepes buffer, pH 7.4. Complexes of siRNA with the DOTAP/DOPE liposomes were prepared at an N/P ratio of 4, by adding an equal volume of radiolabeled siRNA solution to the DOTAP/DOPE liposome dispersion. Complexes of siRNA with RGD-PEG-PEI were prepared at an N/P ratio of 4, by adding an equal volume of radiolabeled siRNA solution to a mixture of RGD-PEG-PEI and branched PEI (in equimolar ratio of ionizable nitrogens).

Formulations of pDNA were prepared in a 20 mM acetate buffer, pH 5.7 containing 20% (g/v) sucrose (Cherng et al., 1999; de Wolf et al., 2005). Complexes with PEI and PEG-PEI were prepared at an N/P ratio of 6. Shortly, radiolabeled pDNA was diluted in acetate buffer containing 40% (g/v) sucrose. To this solution, a linear PEI solution, or a mixture of linear PEI with PEG-PEI (3:1 molar ratio of ionizable nitrogens) in acetate buffer was added. Complexes with p(DMAEA)-ppz were prepared at an N/P ratio of 15, using the same procedure.

2.2.3. Characterization of the complexes

After preparation, the average hydrodynamic diameter and the zeta potential of the complexes were determined using dynamic light scattering and electrophoretic measurements. The size distribution of the complexes was determined using an

ALV CGS-3 system (Malvern Instruments, UK). The ζ-potential of the complexes was determined using a Zetasizer 2000 (Malvern instruments, UK). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The effect of the interaction of the complexes with serum components was tested after incubation in a buffer containing 20 mM Hepes, 0.9% NaCl and 2 mg/ml bovine serum albumin.

2.2.4. Animal experiments

Male A/J mice (5–6 weeks of age) were purchased from Harlan (Horst, The Netherlands). Animal housing and animal experiments were performed according to national regulations and approved by the local animal experiments ethical committee.

A subcutaneous (s.c.) Neuro 2A tumor was induced by inoculation of 1×10^6 mouse neuroblastoma cells (Neuro 2A, ATCC CCL-131) in the flank of each mouse. At an average tumor volume of 500 mm^3, the different radiolabeled nucleic acids formulations were injected into the tail vein of the mice. The injected volume (0.2 ml) corresponded with a radioactivity of >120,000 cpm, and 20 or 30 μg of nucleic acid (for siRNA and pDNA formulations, respectively). At different time points after injection, mice were anesthesized with ether after which blood samples were taken from the vena cava inferior. Subsequently, mice were sacrificed and organs were dissected. Radioactivity of all the samples was determined using liquid scintillation counting, as described earlier (de Wolf et al., 2005). In short, samples were solubilized in Solvable, and subsequently bleached with H_2O_2. Then, samples were incubated in scintillation liquid and measured in a PW4700 scintillation counter (Philips, The Netherlands). Obtained radioactivity levels were corrected for background and for quenching phenomena.

For gene expression studies, 0.2 ml of an unlabeled polyplex dispersion (30 μg pcDNA3Luc/mouse) was administered into the tail vein of the mice. Twenty-four hrs after injection, the mice were sacrificed by cervical dislocation and luciferase levels of the different organs were assessed using a Berthold 9507 Luminometer (EG&G Benelux BV, The Netherlands) as previously described (Verbaan et al., 2003). Transfection efficiency was expressed as RLU/organ, except for tumor tissue (RLU/g). In this particular setting, 6×10^3 RLU corresponded to 1 pg of recombinant luciferase (Promega, The Netherlands).

2.2.5. Statistical analysis

Statistical analysis was performed using GraphPad InStat 3.06. A p-value of < 0.05 was considered to be significant (two-tailed).

3. Results and discussion

3.1. Intravenous delivery of siRNA

We compared circulation kinetics, tumor localization and tissue distribution of two different, radiolabeled siRNA complexes with free radiolabeled siRNA.

3.1.1. Formulations

siRNA against luciferase was radiolabeled with ^{35}S and subsequently complexed with the cationic lipid mixture DOTAP/DOPE or the cationic polymer mixture PEI/RGD-PEG-PEI.

DOTAP is a cationic lipid, commonly used for liposomal transfection (Weyermann et al., 2004; Zhang et al., 2004). Equimolar co-inclusion of the helper lipid DOPE is known to enhance transfection efficiency as it facilitates endosomal destabilization (Xu and Szoka, 1996). Complexes of nucleic acids with DOTAP or DOTAP/DOPE have been successfully applied in several *in vivo* studies (Templeton et al., 1997; Pirollo et al., 2006). Tissue gene expression of cationic lipid/nucleic acid complexes is primarily confined to the liver and the lungs (Fenske et al., 2001; Dass, 2004). Probably, this is a result of the association of the positively charged complexes with negatively charged plasma proteins (e.g. albumin, complement proteins) which leads to the facilitated uptake of the complexes by the non-parenchymal cells within the liver. In addition, interaction of the cationic complexes with erythrocytes and plasma proteins may favor the formation of aggregates in the circulation. These aggregates can become entrapped in the lung capillaries, promoting uptake by lung endothelial cells (Mclean et al., 1997; Sakurai et al., 2001).

Schiffelers et al. used siRNA complexes consisting of a mixture of PEI and RGD-PEG-PEI (PEI to which is grafted $PEG_{3.4\,kDa}$, distally modified with an RGD peptide), for therapeutic application in mice. The incorporation of a PEG moiety within the complexes is supposed to enable shielding of the cationic surface charge of siRNA complexes, thereby limiting the interaction with plasma proteins and erythrocytes. The incorporation of the RGD-motif allowed the targeting of the complexes towards the $\alpha_v\beta_3$ integrins, overexpressed on tumor angiogenic endothelial cells, but also present on macrophages (Schraa et al., 2002). Intravenous administration of these complexes led to tumor growth inhibition in different tumor models (Schiffelers et al., 2004a).

Positively charged, nanoscale particles, suitable for intravenous injection were formed upon complexation of the negatively charged siRNA with the two different carrier types (Table 1). The RGD-PEG-PEI complexes exhibited a much lower zeta potential than observed for the DOTAP/DOPE complexes, reflecting the shielding effect of the PEG-component incorporated in this formulation.

Table 1
Mean particle size and zeta potential of siRNA complexes with DOTAP/DOPE or RGD-PEG-PEI, at the given N/P ratio

	siRNA DOTAP/DOPE complexes	siRNA RGD-PEG-PEI complexes
Mean particle size (nm)	250 ± 10	130 ± 10
Mean zeta potential (mV)	39 ± 2	8 ± 2
N/P ratio	4	4

Data are presented as the mean ± S.D. of three measurements. Polydispersity values were within acceptable limits (<0.35). The N/P ratio was defined as the molar excess of the ionizable nitrogens of the cationic carrier (N) to the phosphate groups of the siRNA (P).

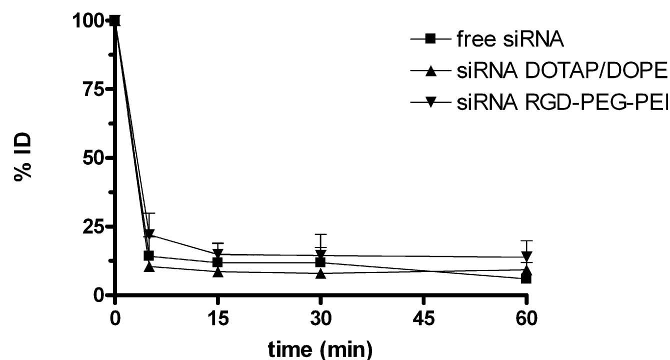


Fig. 1. Circulation kinetics of free ^{35}S-labeled siRNA, and complexes of ^{35}S siRNA with DOTAP/DOPE or RGD-PEG-PEI, following i.v. administration in A/J mice bearing a s.c. Neuro 2A tumor (20 μg siRNA/mouse). Data are expressed as mean percentage of injected dose (% ID) ± S.D. (n = 3).

Table 2
Tumor accumulation of free ^{35}S-labeled siRNA, and complexes of ^{35}S siRNA with DOTAP/DOPE or RGD-PEG-PEI, 15 and 60 min after i.v. administration in A/J mice bearing a s.c. Neuro 2A tumor (20 μg siRNA/mouse)

Formulation	Tumor localization (% ID/g)	
	15 min	60 min
Free siRNA	3.0 ± 1.8	2.3 ± 0.5
siRNA DOTAP/DOPE	2.5 ± 0.8	2.9 ± 1.7
siRNA RGD-PEG-PEI	4.1 ± 1.7	5.2 ± 2.9

Data are expressed as mean percentage of injected dose per gram tumor (% ID/g) ± S.D. (n = 3). Average tumor weight was 450 mg.

3.1.2. Circulation kinetics, tumor localization and tissue distribution

We determined the circulation kinetics, tumor localization and tissue distribution of the radiolabeled siRNA, following intravenous injection of the different formulations into A/J mice bearing a s.c. Neuro 2A tumor.

Upon intravenous administration, the blood levels of free siRNA exhibited a clearance pattern characterized by a fast distribution phase and a subsequent slower elimination phase (Fig. 1). Five min after injection, only 14 ± 7% of the injected dose resided in the bloodstream, in good agreement with literature (Braasch et al., 2004; Van De Water et al., 2006). Upon complexation with the cationic carriers, a similar biphasic elimination profile was found. Complexation of siRNA with either DOTAP/DOPE or RGD-PEG-PEI did not significantly affect siRNA blood levels at any of the time points. The poor circulation profile of the latter complexes was unexpected in view of the shielded character of the complexes. Apparently, the PEG-component within the RGD-PEG-PEI formulation did not provide sufficient steric stabilization to prolong the blood circulation of siRNA or was overshadowed by the enhanced uptake as a result of the displayed RGD-peptide (Schraa et al., 2002).

The amount of radioactivity recovered from the tumor after intravenous administration of the radiolabeled siRNA complexes is given in Table 2. Sixty minutes after injection, 2% of the injected free siRNA dose had accumulated per gram tumor tissue. Surprisingly, complexation with DOTAP/DOPE did not significantly increase siRNA tumor levels at the different time points. The same held true for siRNA complexed with RGD-PEG-PEI, despite the proven ability of RGD-complexes to target towards the tumor neovasculature. Apparently, the rapid clearance of the complexes hindered tumor accumulation. We conclude that other mechanisms must be taken into account to explain the additional value of siRNA delivery systems for intravenous administration, likely due to carrier-induced changes in the siRNA intratumoral distribution. Fig. 2 illustrates how both siRNA formulations localize differently, within a slice of the tumor border (Fig. 2A and B). We found the RGD-PEG-PEI complexes to be preferentially localized within the viable rim of the tumor tissue, whereas DOTAP/DOPE complexes could be visualized throughout the whole tumor tissue. The tumor periphery contains the areas of active angiogenesis featured by the highest number of small capillaries, which are specifically overexpressing the $\alpha_v\beta_3$ integrin (Hu et al., 2003).

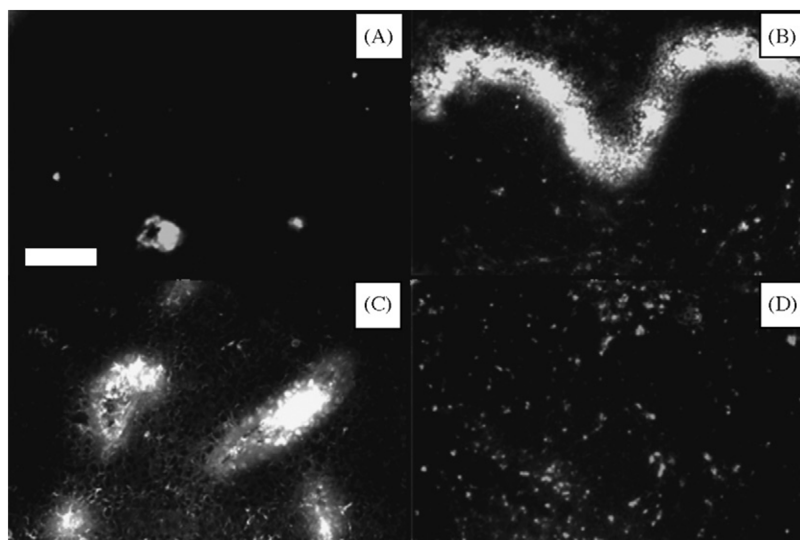


Fig. 2. Micrographs of a Neuro 2A tumor tissue (A and B) and spleen (C and D) isolated from mice 60 min after i.v. administration in A/J mice (40 μg siRNA/mouse). siRNA formulations were DOTAP/DOPE complexed FITC-labeled siRNA (A and C) or RGD-PEG-PEI complexed FITC-labeled siRNA (B and D) (bar = 250 μm).

Earlier, using fluorescence images, Schiffelers et al. reported on increased tumor localization over free siRNA, when using siRNA complexes of RGD-PEG-PEI (Schiffelers et al., 2004a). These contradictive findings may result from the fact that in the latter study, excised tumor tissue was visualized macroscopically from the outside, inherently focusing on fluorescence in the tumor periphery only.

The tissue distribution of the radiolabeled siRNA formulations is presented in Fig. 3. Tissue levels of free siRNA were low and primarily confined to the kidney and the liver (5.2 and 3.9% ID at 15 min post-injection (p.i.), respectively). This finding suggests a rapid renal clearance by glomerular filtration. The substantial liver accumulation of unformulated siRNA is attributed to uptake by non-parenchymal liver cells (Nolting et al., 1997). The tissue distribution of siRNA complexed with DOTAP/DOPE was also characterized by a limited tissue deposition. Highest tissue levels were found within the liver, the lung and the kidney (16, 2.6 and 4.0% ID, at 15 min p.i., respectively). The rapid clearance of cationic lipid nucleic acid complexes, together with their preferential accumulation in the 'first pass' organs is attributed to their interaction with blood components, as a result of their chemical and cationic nature (Fenske et al., 2001). The significant kidney localization is likely the result of glomerular filtration of siRNA (fragments), released upon disintegration of the complexes. siRNA complexed with RGD-PEG-PEI preferentially accumulated in the liver (36% ID, at 15 min p.i.). Significant deposition was observed as well in the lung, the kidney and the spleen (7.0, 3.6 and 3.4% ID, at 15 min p.i., respectively). The relatively high degree of hepatosplenic localization is probably a result of the RGD-mediated targeting of the complexes to $\alpha_v\beta_3$ integrins, expressed by the macrophages (Martinez-Pomares et al., 1996).

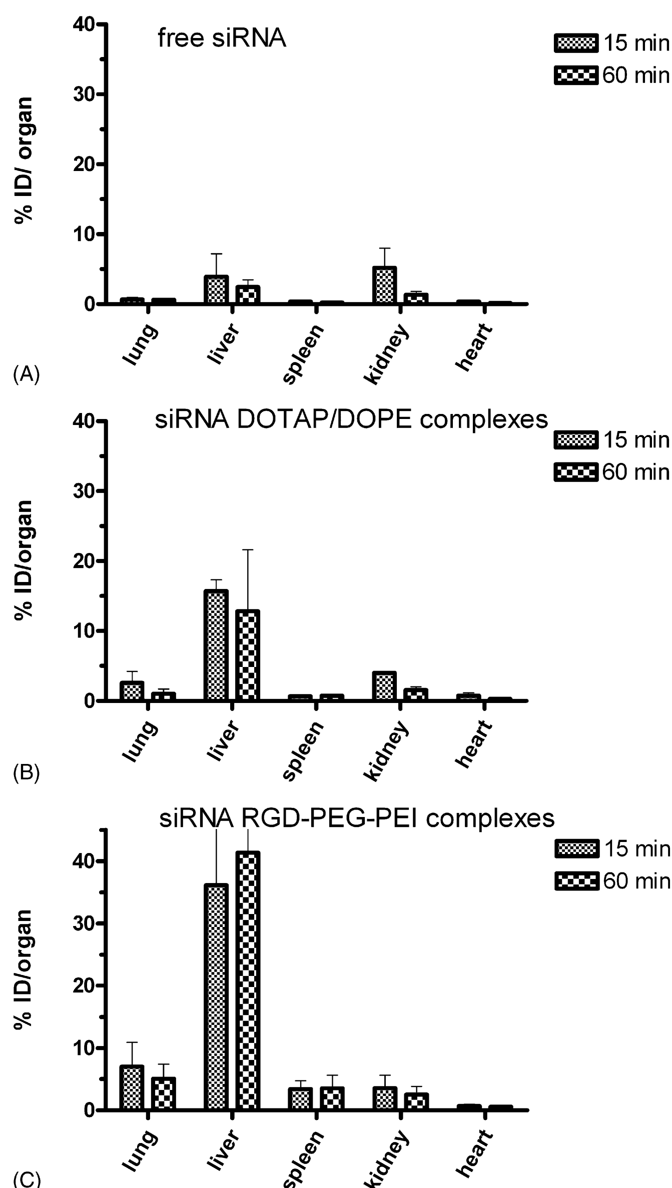


Fig. 3. Tissue distribution of free ^{35}S-labeled siRNA (A), and complexes of siRNA with DOTAP/DOPE (B) or RGD-PEG-PEI (C), 15 and 60 min after i.v. administration in A/J mice bearing a s.c. Neuro 2A tumor (20 μg siRNA/mouse). Data are expressed as mean percentage of injected dose per organ (% ID/organ) ± S.D. ($n = 3$).

Fig. 2C and D are micrographs of the spleen, illustrating the differences in intraorgan distribution of the siRNA delivery systems. Accumulation of the DOTAP/DOPE complexes was confined to the red pulpa of the spleen, indicating efficient filtration of larger particles or aggregates and subsequent recognition by macrophages. The RGD-PEG-PEI complexes were more homogeneously distributed over the whole tissue, suggesting prevention of aggregate formation through PEG-shielding and receptor-mediated uptake by macrophages stimulated by the RGD-peptide.

3.2. Intravenous delivery of plasmid DNA

We compared circulation kinetics, tumor localization and tissue distribution of different radiolabeled pDNA complexes.

3.2.1. Formulations

pDNA was radiolabeled with ^{35}S and subsequently complexed with different polymeric cationic carrier systems. We compared complexes based on the linear polymer polyethyleneimine (PEI) with complexes based on PEGylated linear PEI (PEG-PEI), and complexes based on the polymer poly(2-dimethylamino ethylamino)phosphazene (p(DMAEA)-ppz).

Linear PEI is one of the most effective cationic polymers for the delivery of pDNA. It combines a strong plasmid condensing ability with superior transfection efficiency (Lungwitz et al., 2005; Park et al., 2006). Intravenous administration of cationic PEI polyplexes into mice bearing a s.c. Neuro 2A tumor yields considerable tumor gene expression (Kircheis et al., 1999). However, the cationic character of PEI complexes promotes their interaction with plasma proteins and erythrocytes, enhancing their clearance by non-target tissues (Zou et al., 2000; Lungwitz et al., 2005). The use of PEG-PEI (PEI to which polyethylene-glycol (PEG) is grafted), is aimed to shield the positive surface charge of the polyplexes (Wolschek et al., 2002; Kursa et al., 2003). As a result, application of PEG-PEI polyplexes in tumor bearing mice yields a more tumor selective gene expression profile compared to PEI polyplexes, as is

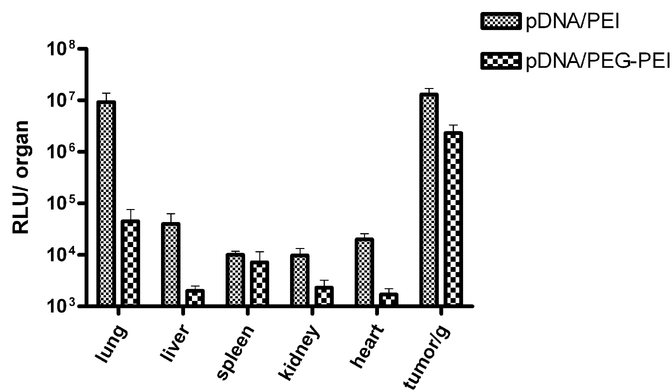
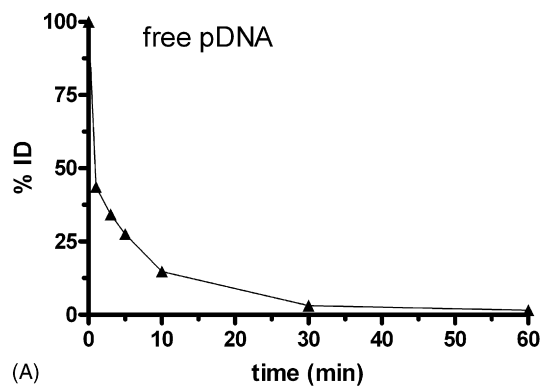


Fig. 4. Luciferase gene expression, 24 h after i.v. administration of pDNA complexes with PEI and PEG-PEI into A/J mice bearing a s.c. Neuro 2A tumor (30 μg pDNA/mouse). Luciferase expression is plotted as relative light units per organ (RLU/organ) except for tumor tissue (RLU/g) ± S.D. ($n = 3$). Average tumor weight was 450 mg.



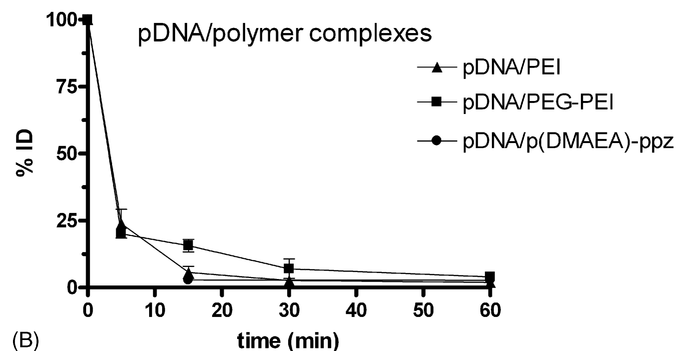


Fig. 5. Circulation kinetics of free ^{32}P-labeled pDNA and ^{35}S-labeled pDNA/polymer complexes, following i.v. administration in mice (30 μg pDNA/mouse). Free pDNA in Balb/c mice (A, Verbaan et al., 2003), and pDNA/polymer complexes (B). pDNA/polymer complexes used were pDNA/PEI, pDNA/PEG-PEI, or pDNA/p(DMAEA)-ppz. Data are expressed as mean percentage of injected dose (% ID) ± S.D. ($n = 3$).

illustrated in Fig. 4. The increased selectivity of gene expression towards the tumor tissue in the case of PEG-PEI polyplexes has been related to the reduced uptake by non-target tissue, as a result of their shielded character. Moreover, the extended blood circulation of shielded polyplexes might enable an enhanced accumulation within the tumor based on the so-called enhanced permeability and retention (EPR) effect (Maeda et al., 2000; Ogris and Wagner, 2002). Based on this principle, the application of long-circulating gene delivery vectors has resulted in a passive tumor accumulation of 3.5–10% of the injected dose (Fenske et al., 2001; Kaul and Amiji, 2004; Verbaan et al., 2004). Tumor growth inhibition has been demonstrated in different mouse tumor models, using PEGylated transferrin-PEI polyplexes (Kircheis et al., 2002; Kursa et al., 2003).

We recently reported on the application of the biodegradable polymer p(DMAEA)-ppz, as a pDNA carrier in tumor bearing mice (Luten et al., 2003; de Wolf et al., 2005). Compared to unshielded PEI complexes, the cationic p(DMAEA)-ppz complexes demonstrated more selective tumor gene expression after intravenous administration.

Complexation of the pDNA resulted in positively charged particles of approximately 100 nm (Table 3). The zeta potential of the PEG-PEI polyplexes was low compared to the PEI complexes as a result of the shielding effect of PEG (9 and 31 mV, respectively). The addition of albumin resulted in a drop of the zeta potential values of all formulations to around 0 mV, which is in good agreement with earlier findings on PEI, p(DMAEA)-ppz and PLL complexes (de Wolf et al., 2005; Dash et al., 1999). The mean size of the complexes increased upon incubation with albumin, most pronouncedly in the case of the unshielded formulations (data not shown). Possibly, albumin induced aggregate formation is sterically hindered by the PEG chains, present on the surface of the shielded PEG-PEI complexes.

3.2.2. *Circulation kinetics, tumor localization and tissue distribution*

We compared the circulation kinetics, tumor localization and tissue distribution of the various radiolabeled pDNA complexes following intravenous injection of the different formulations into A/J mice bearing a s.c. Neuro 2A tumor. Data were compared with data on free pDNA, obtained in earlier experiments performed by our group (Verbaan et al., 2003).

Fig. 5 shows the blood circulation profiles of the injected formulations. Free pDNA showed a rapid pDNA clearance. Ten and 30 min after injection, only 15 and 3% of the injected dose resided in the bloodstream, respectively. The different

Table 3
Mean particle size and zeta potential of pDNA/polymer complexes at the given N/P ratio

	pDNA/PEI complexes	pDNA/PEG-PEI complexes	pDNA/p(DMAEA)-ppz complexes
Mean particle size (nm)	110 ± 20	100 ± 20	100 ± 10
Mean zeta potential (mV)	31 ± 6	9 ± 3	37 ± 3
N/P ratio	6	6	15

Data are presented as the mean ± S.D. of three measurements. Polydispersity values were within acceptable limits (<0.35). The N/P ratio was defined as the molar excess of the ionizable nitrogens of the cationic carrier (N) to the phosphate groups of the pDNA (P).

Table 4
Tumor accumulation of ^{35}S-labeled pDNA/polymer complexes, 15 and 60 min after i.v. administration into A/J mice bearing a s.c. Neuro 2A tumor (30 μg pDNA/mouse)

	15 min	60 min
pDNA/PEI complexes	2.7 ± 0.6	0.7 ± 0.1
pDNA/PEG-PEI complexes	1.9 ± 1.0	1.7 ± 0.6
pDNA/p(DMAEA)-ppz complexes	1.5 ± 0.8	1.0 ± 0.6

pDNA was complexed with PEI, PEG-PEI or p(DMAEA)-ppz. Data are expressed as mean percentage of injected dose per gram tumor (% ID/g) ± S.D. (n = 3). Average tumor weight was 600 mg.

unshielded, positively charged complexes were subjected to a similar or even faster clearance pattern (5.7 and 2.9% ID at 15 min p.i., for PEI and p(DMAEA)-ppz polyplexes, respectively). The incorporation of PEG-PEI into the polyplexes slightly reduced the pDNA clearance (blood levels were two- to three-fold higher, at 15–60 min p.i.). The PEG-mediated prolongation of polyplex blood levels is relatively small compared to earlier findings reported for liposomes (Woodle and Lasic, 1992). Apparently, the PEG-PEI polyplexes are still prone to efficient opsonization. This finding is in good agreement with an earlier report on the PEGylation of branched PEI-polyplexes, applied at a similar DNA dose (Merdan et al., 2005). To our knowledge, only three groups have demonstrated a significant prolongation of pDNA blood levels using cationic polymers (>20% ID, at 30 min p.i.). Remarkably, in all three cases, PEG was coupled to the surface of the polyplexes subsequent to polyplex formation (Ogris et al., 1999; Ward et al., 2002; Verbaan et al., 2004).

pDNA radioactivity levels in the tumor tissue are given in Table 4. In all cases, a limited tumor accumulation of 1% of the injected dose per gram tumor was observed at 60 min p.i., similar to the tumor localization levels reported earlier (de Wolf et al., 2005). No significant differences could be demonstrated, comparing the different polyplex systems at the two time points. Apparently, the somewhat prolonged blood circulation of the PEG-PEI polyplexes over the PEI polyplexes did not result in an increase in pDNA tumor accumulation. From these data, we conclude that the tumor selective gene expression observed in the case of PEG-PEI (Fig. 4) and p(DMAEA)-ppz complexes (de Wolf et al., 2005) is not driven by preferential accumulation of the pDNA in the tumor.

We examined the pDNA tissue distribution of the different formulations (Figs. 6 and 7). Uncomplexed pDNA preferentially accumulated in the liver and the kidney, in good agreement with earlier literature (Kawabata et al., 1995). As compared to free pDNA, all polyplex systems demonstrated a low kidney uptake, reflecting a high level of colloidal stability within the bloodstream. The unshielded pDNA complexes showed pronounced uptake by the organs of the reticuloendothelial system (liver and spleen), which is in good agreement with earlier literature (Ogris and Wagner, 2002) and is the likely cause of their efficient blood

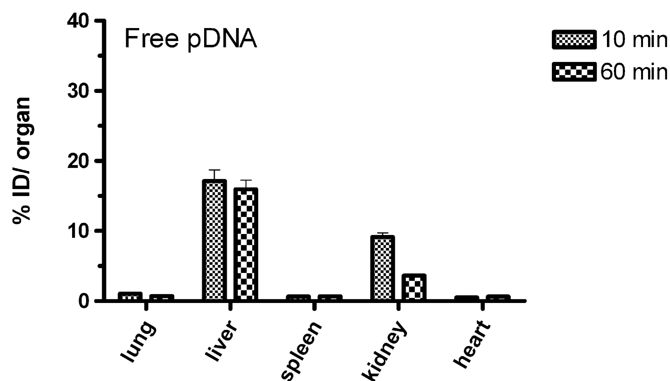


Fig. 6. Organ distribution of free, ^{32}P-labeled pDNA, 10 and 60 min after i.v. administration in Balb/c mice (30 μg pDNA/mouse). Data are expressed as mean percentage of injected dose per organ (% ID/organ) ± S.D. (n = 3) (Verbaan et al., 2003).

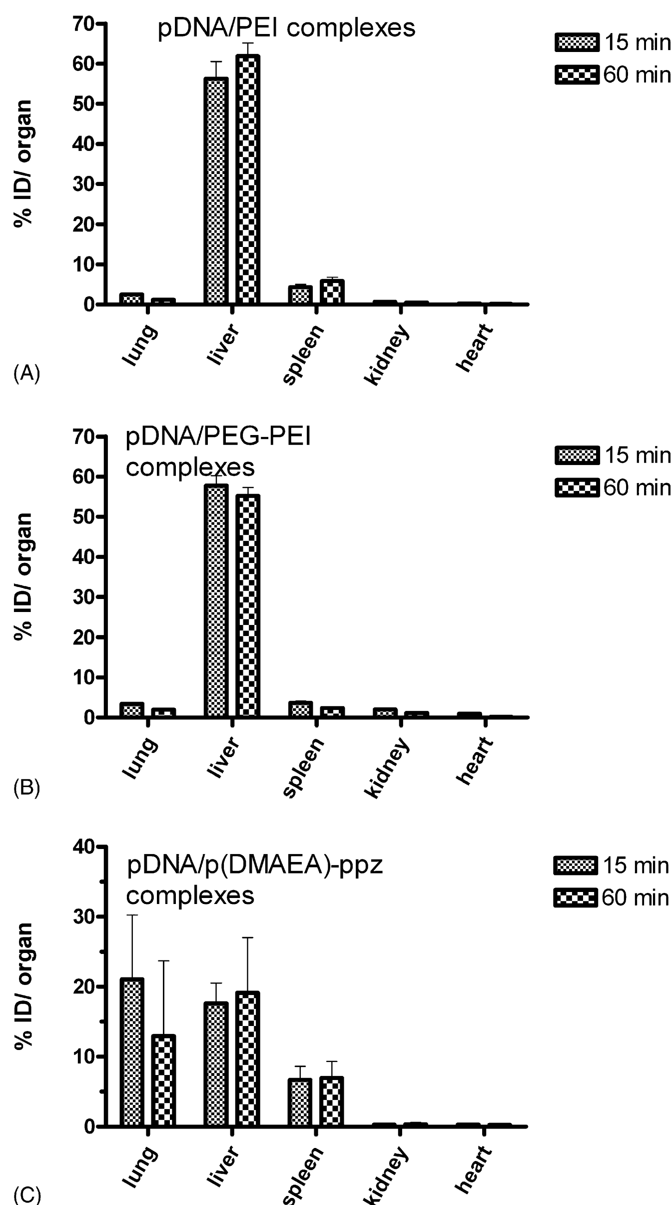


Fig. 7. Organ distribution of ^{35}S-labeled pDNA/polymer complexes, 15 and 60 min after i.v. administration in A/J mice bearing a s.c. Neuro 2A tumor (30 μg pDNA/mouse). pDNA/PEI complexes (A), pDNA/PEG-PEI complexes (B), and pDNA/p(DMAEA)-ppz complexes (C). Data are expressed as mean percentage of injected dose per organ (% ID/organ) ± S.D. (n = 3).

clearance (Fig. 7A and C). The slightly increased circulation time of the PEG-PEI polyplexes did not affect pDNA uptake in these organs (Fig. 7B). The p(DMAEA)-ppz based polyplexes demonstrated a more pronounced lung accumulation compared to PEI polyplexes. Earlier, we related the high lung accumulation of p(DMAEA)-ppz polyplexes with their tendency to interact with erythrocytes (de Wolf et al., 2005).

The observed pDNA tissue distribution levels induced by the different polyplexes (Fig. 7) do not correlate with their respective gene expression levels (Fig. 4, de Wolf et al., 2005). Therefore, the degree of localization in a particular tissue is apparently not predictive for the degree of gene expression. Rather, differences in tissue gene expression induced by different transfection systems are determined by factors such as differences in distribution of the extravasated complexes over the tissue, and nuclear entry determinants such as intracellular processing and rate of cellular proliferation.

4. Conclusion

In the present study, we compared the circulation kinetics, tumor localization and tissue distribution of different nucleic acid complexes. The delivery systems included all have demonstrated antitumor activity or significant tumor reporter gene expression in earlier studies. Intravenously administered, free siRNA demonstrated poor circulation kinetics, together with a limited accumulation in the tumor tissue, the liver and the kidneys. Although complexation of siRNA with the two carriers clearly affected its tissue distribution profile, it did not prolong siRNA blood circulation. Efficient opsonization, complex desintegration, or active targeting towards macrophages are likely to contribute to the high blood clearance rate. The coupling of an RGD peptide, enabling the targeting of siRNA complexes towards neovascularized tumor capillaries, did not lead to an enhanced tumor accumulation. However, within the tumor tissue, siRNA complexes did selectively distribute towards the targeted tumor endothelial cells present in the tumor periphery. The condensation of pDNA with different unshielded polymeric carriers did not improve pDNA blood clearance and tumor localization. Accumulation of the polyplexes was observed mainly in the organs of the reticuloendothelial system, in good agreement with earlier literature. The use of PEG, to shield the surface of the PEI polyplexes, yielded a slightly extended blood circulation time of the complexes. However, the increased blood residence time was not paralleled by an enhanced accumulation of pDNA in the tumor.

In conclusion, our data do not point to significant improvements of circulation kinetics and tumor accumulation due to the use of the carrier systems studied here. Therefore, the value of the use of carrier-mediated intravenous delivery of nucleic acids cannot be solely attributed to benefits relevant during the transport of the complexes to the tumor. Rather, the carrier mediated benefits are arising from changes in the intratumoral fate of the nucleic acids conferred by the carrier systems, like e.g. the targeting to certain tumor components and the improvement of the intracellular targeting process.

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