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Research Paper

Evaluation of Pharmacokinetics of Bioreducible Gene Delivery Vectors by Real-time PCR

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Purpose. To investigate pharmacokinetics of reversibly stabilized DNA nanoparticles (rSDN) using a single-step lysis RT-PCR.
Methods. rSDN were prepared by coating bioreducible polycation/DNA polyplexes with multivalent *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers. Targeted polyplexes were formulated by linking cyclic RGD ligand (c(RGDyK)) to the HPMA surface layer of rSDN. The pharmacokinetic parameters in tumor-bearing mice were analyzed by PKAnalyst®.
Results. The pharmacokinetics of naked plasmid DNA, simple DNA polyplexes, rSDN, and RGD-targeted rSDN exhibited two-compartment model characteristics with area under the blood concentration–time curve (AUC) increasing from 1,102 $ng \cdot ml^{-1} \cdot min^{-1}$ for DNA to 3,501 $ng \cdot ml^{-1} \cdot min^{-1}$ for rSDN. Non-compartment model analysis revealed increase in mean retention time (MRT) from 4.5 min for naked DNA to 22.9 min for rSDN.
Conclusions. RT-PCR is a sensitive and convenient method suitable for analyzing pharmacokinetics and biodistribution of DNA polyplexes. Surface stabilization of DNA polyplexes can significantly extend their MRT and AUC compared to naked DNA. DNA degradation in rSDN in blood circulation, due to a combined effect of disulfide reduction and competitive reactions with charged molecules in the blood, contributes to DNA elimination.

KEY WORDS: gene delivery; pharmacokinetics; real-time PCR; tumor targeting.

INTRODUCTION

Systemic targeted delivery of therapeutic nucleic acids remains a major challenge compromising successful application of gene therapy. An efficient gene delivery vector is expected to be inert in the extracellular space and undergo controlled activation leading to the release of therapeutic gene upon reaching the intracellular space. The extracellular space is predominantly oxidizing and the intracellular space is enriched with high concentrations of redox molecules like glutathione (GSH) and various redox enzymes (1). The intracellular selectivity is primarily determined by the approximately 1,000-fold difference between the cytosolic and plasma concentrations of reduced GSH (2). The cellular distribution of GSH suggests that intracellular reduction proceeds mainly in the cytoplasm and nucleus (3–6). This high redox potential gradient existing across the biological membranes can be exploited for designing carriers by incorporating disulfide bonds in the vector.

Reversibly stabilized DNA nanoparticles (rSDN) display good stability and tumor targeting ability *in vitro* (7, 8). The rSDN nanoparticles have a bioreducible core coated with a hydrophilic multivalent polymer to which targeting ligands can be easily attached. The bioreducible core consists of a complex of DNA with high molecular weight reducible polycation prepared by a polymerization of short cationic blocks linked by disulfide bonds.

Evaluation of pharmacokinetics (PK) is an important part of a new drug development. PK analysis of most small molecule drugs relies on quantification of the drug in blood and tissues using LC–MS/MS methods (9–12). Unfortunately, the upper molecular weight limit of LC–MS is about 2,000 *m/z* and thus this technique is not suitable for analysis of DNA-based therapeutics due to large molecular weight of the typical gene constructs used ($4–6 \times 10^6$ Da) (13). The following three methods were reported to be used for quantifying DNA *in vivo*: radiolabeling of DNA (14–16), gel electrophoresis (17) and competitive PCR. The use of radiolabeled DNA suffers from its inability to distinguish between functional intact DNA and partially or fully degraded DNA. Gel electrophoresis, while suitable to measure intact DNA *in vivo*, is hindered by its low detection limit ~0.25 $\mu g\ ml^{-1}$ (17). Other reports used competitive and quantitative PCR to measure PK of DNA

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ABBREVIATIONS: AUC, Area under the blood concentration–time curve; GSH, Glutathione; HPMA, *N*-(2-hydroxypropyl) methacrylamide; LC–MS, Liquid chromatography–mass spectroscopy; MRT, Mean retention time; PAA, Poly-L-aspartic acid; PK, Pharmacokinetics; PLL, Poly-L-lysine; RGD, Arginine–glycine–aspartic acid; rPLL, Reducible poly-L-lysine; rSDN, Reversibly-stabilized DNA nanoparticles, i.e. rPLL/DNA complexes coated with HPMA copolymer; RT-PCR, Real-time polymerase chain reaction.

polyplexes (18, 19). Competitive PCR is based on co-amplification of the sample template together with various amounts of internal standard competitor, sharing with the target the primer recognition sites, but differing in size. The competitor is constructed to share the same sense and antisense primer used for target amplification (20). This method has a lot of advantages such as no need for DNA labeling and high sensitivity compared with the above two methods. However, it requires time consuming DNA extraction and the use of gel electrophoresis to verify the amount of DNA amplified in the PCR reaction, thus falling short of the potential accuracy and easy automation.

The objective of the present study was to investigate PK of bioreducible rSDN using a reliable and easily scalable real time PCR (RT-PCR) method that would not require time consuming tissue extraction of DNA.

MATERIALS AND METHODS

Materials

Copolymer of HPMA with 9.3 mol% of methacryloyl-glycylglycine 4-nitrophenyl ester (M_w 4.1×10^4) was synthesized as described previously (21). Reducible PLL (rPLL, M_w 3.0×10^4) was synthesized by polymerization of $CK_{10}C$ peptide in 50% dimethylsulfoxide in HEPES buffer (pH 7.4) using previously described general protocol (8). Average molecular weights of the synthesized polymers were determined by size exclusion chromatography equipped with a refractive index detector and multi-angle laser light scattering detector using either CATSEC-300 (rPLL) or Polymer Labs PL gel 5 μm mixed C column (HPMA copolymer). Targeting peptide cyclic RGDyK was purchased from Peptides International, Inc. (Louisville, KY). gWiz™ High-Expression Luciferase (gWIZLuc) plasmid was purchased from Aldevron. RRT-PCR lysis buffer was made of 0.5 mg ml^{-1} Proteinase K, 2 mg ml^{-1} poly-L-aspartic acid (Sigma, molecular weight 35,000) and 10 mM tris-hydroxypropyl-phosphine (EMD Biosciences). The real time PCR was performed in ABI Prism® 7300. The following probe and primers of gWIZLuc were designed in BioSearch online RealTimeDesign™ software: FAM-BHQ probe TCAGGATTACAAGATTCAAAGTGCGCT, forward primer GAAGAGCTGTTTCTGAGG, reverse primer CGAAGAAGGAGAATAGGGT. The master mix was purchased from Eurogentec (cat. # RT-CKFT-18S, RT-QP2X-03). DNase was from Promega® (Cat. No. M610).

Formulation of Polyplexes

rPLL/DNA polyplexes were prepared by fast addition of rPLL solution to DNA solution to achieve N:P ratio 2 and final DNA concentration 32 μg ml^{-1} (7). The polyplexes were immediately vortexed for 30 s and incubated for 30 min before use. To coat the rPLL/DNA polyplexes, HPMA copolymer solution was added to the polyplex solution to achieve final copolymer concentration 2 mg ml^{-1}. The pH of the reaction mixture was adjusted to 7.8 with 500 mM HEPES buffer as previously described (7). RGD-targeted polyplexes (RGD-rSDN) were prepared by including 200 μg ml^{-1} of the respective peptide c(RGDyK) in the coating reaction. Unreacted polymer and peptides were removed after 24 h reaction at room temperature by VivaSpin20 centrifugal concentrator (molecular weight cut-off 1×10^5). The DNA recovery after purification was measured by gel electrophoresis after incubating the polyplexes with 20 mM DTT and 2 mg ml^{-1} PAA to ensure complete release of DNA. DNA recovery after polyplex purification was typically >90% (22). The content of RGD was determined by amino acid analysis. RGD content in RGD-rSDN was determined to be 660±20 RGD molecules for every DNA molecule ($n=3$) by amino acid analysis from the content of tyrosine (Texas A&M University Protein Chemistry Lab).

RT-PCR

Calibration curve was constructed using luciferase plasmid DNA diluted to 20 μg ml^{-1}, 2 μg ml^{-1}, 200 ng ml^{-1}, 20 ng ml^{-1}, 2 ng ml^{-1}, and 0.2 ng ml^{-1} standard solutions. Two microliters of the standard was added to 25 μl of PCR lysis buffer, incubated at 25°C overnight, 1× DNase-free TB buffer was added to 400 μl, and solution heated at 95°C for 10 min. Five microliters of the sample and 20 μl of master mix with Taqman probe were added to PCR tube to perform RT-PCR. During the RT-PCR cycle, 5 μl of DNA template, 500 nM probe and 100 nM primer pairs were added. The PCR cycle was run for 2 min at 50°C, 10 min at 95°C, 40 cycles for 15 s at 95°C, and 1 min at 60°C. Cycle of threshold (Ct) was determined. Every sample was measured in triplicate. For validation, standard curves of DNA, polyplex, rSDN and RGD-rSDN were measured with or without addition of 2 μl blood before 37°C incubation.

Blood and Tissue Collection

All animal research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). The animal studies were performed in male C57/B6 mouse (8 weeks old) bearing B16F10 melanoma tumors. The animals were inoculated with 1×10^5 B16F10 cells in left hind flank zone (23, 24). The animals received a single tail injection of 1 μg DNA/mouse (20 μg ml^{-1}, 50 μl), polyplex, rSDN and RGD-rSDN (Fig. 1). In the PK analysis, blood sample was obtained using tail vein venipuncture and 2 μl blood was withdrawn with a 2 μl quantitative capillary at different time points. For naked plasmid DNA and polyplex, the blood was collected at 0.5, 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 25, 30 and 40 min post-injection. For rSDN and RGD-rSDN, blood was collected at 0.5, 1, 2.5, 5, 7.5, 10, 15, 20, 30, 40, 60, 90 and 120 min post-injection. Immediately after collection, 2 μl of the blood was mixed with 25 μl of PCR lysis buffer and incubated at 37°C for 12 h, after which DNase-free 1× TB buffer was added up to 400 μl and the sample was heated at 95°C for 10 min and 5 μl of that sample was analyzed for plasmid DNA content by RT-PCR. Every PK experiment with a single formulation was performed using six mice.

For tissue distribution studies, animals were sacrificed 1, 2, and 3 h after tail-vein injection and organs were harvested. To minimize the influence of plasmid DNA in blood, the tissue samples were washed thoroughly several times with saline, blotted dry and weighed. The samples were then suspended in PCR lysis buffer at a concentration of 50 mg of

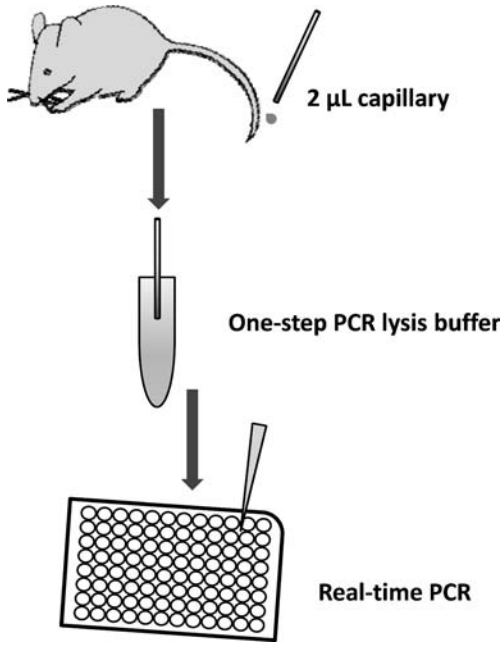


Fig. 1. Evaluating pharmacokinetics of polyplexes by RT-PCR. Mice are dosed i.v. with 50 µl of 20 µg DNA ml^{-1} of the delivery vectors. Two microliter blood samples are withdrawn with a quantitative capillary and immediately mixed with 25 µl of a modified one step PCR lysis buffer and content of intact plasmid DNA is quantified with RT-PCR.

tissue per milliliter and homogenized using a Tissuemiser Homogenizer in an ice bath. Immediately after homogenization, 2 µl of homogenized solution was mixed with 25 µl PCR lysis buffer and incubated in 37°C for 12 h, DNase-free 1× TB buffer was added up to 400 µl and sample was heated at 95°C for 10 min and 5 µl of that sample was analyzed for plasmid DNA content by RT-PCR. DNA concentration <0.1 ng ml^{-1} was considered as non-detectable. Every distribution experiment with each formulation was performed using six mice.

Blood concentration of DNA, polyplex, rSDN, RGD-rSDN was analyzed by PKAnalyst® Software (Micromath Research) using two compartment and non-compartment model.

DNA Degradation in Fresh Whole Blood

Fresh blood was taken from tumor-bearing mice and stabilized by 10 IU ml^{-1} heparin or 3.8 mg ml^{-1} sodium citrate. Each gene delivery vector was mixed with fresh blood and DNA concentration was measured by RT-PCR at the following time points: 0.5, 10, 20, 40, 60, 90, 120, 150, 180, 240, 300, 360 min. The measurements were made in quadruplicate.

DNA Release from Different Formulations in the Presence of Polyelectrolyte

The release of polyplexes, rSDN, RGD-rSDN against polyelectrolyte exchange reactions with PAA (MW 35,000) was evaluated by monitoring the release of free DNA using agarose gel electrophoresis. The polyplexes, rSDN, RGD-rSDN were incubated with 1 mg ml^{-1} PAA at 37°C for 3 h analyzed on 0.8% agarose gel with 50 µl of Ethidium Bromide (1 mg ml^{-1}) added to 100 ml gel.

Effect of Glutathione and Polyanions on DNA Degradation in DNase Buffer *In Vitro*

The gene delivery vectors were mixed with DNase buffer (10 U DNase ml^{-1} Promega® Cat. No. M610) with or without 0.5 mM GSH or 0.5 mg ml^{-1} poly-L-aspartic acid. DNA concentration was measured by RT-PCR at different time points: 0, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 30, 45, 60 min. Every experiment was performed four times. Data were analyzed using first-order kinetic equation: $dC_{DNA} = k_d \times C_{DNA} \times dt$. Degradation constant k_d was determined from the slope of the plot of ln(C) *vs.* time, and $t_{1/2}$ was calculated as $t_{1/2}=0.693/k_d$.

Statistical Analysis

Statistical significance of the results was evaluated using two-tailed heteroscedastic Student's t-test in Microsoft excel. A p-value of <0.01 was considered significant.

RESULTS AND DISCUSSION

Validation of the RT-PCR Methodology for Quantification of DNA in rSDN

Successful use of RT-PCR for quantification of DNA in rSDN requires quantitative disassembly of the polyplexes. Number of commercial one-step buffers exist that can be used for RT-PCR. However, none of the available lysis buffers could quantitatively release pDNA from the rSDN and even simple rPLL/DNA polyplexes, rendering them unsuitable for

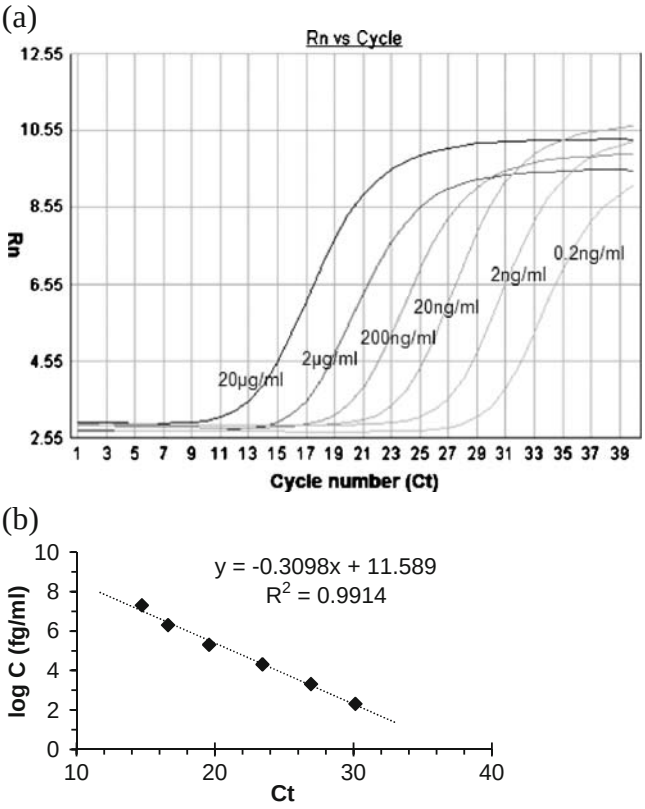
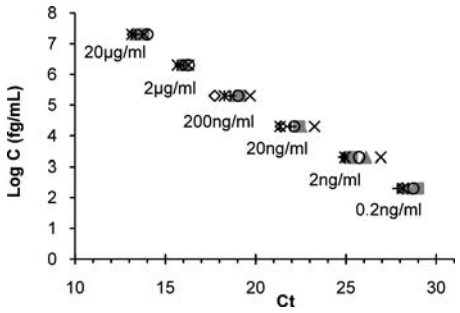


Fig. 2. Calibration of RT-PCR. Typical amplification curves (**a**) and luciferase plasmid standard curve (**b**).



Sample	Linear fit log C = -XCt + Y	R
DNA	X = -0.328, Y = 11.43	0.990
DNA + blood	X = -0.325, Y = 11.62	0.996
rPLL/DNA	X = -0.317, Y = 11.42	0.996
rPLL/DNA + blood	X = -0.322, Y = 11.68	0.987
rSDN	X = -0.328, Y = 11.45	0.995
rSDN + blood	X = -0.331, Y = 11.75	0.995
RGD-rSDN	X = -0.340, Y = 11.74	0.998
RGD-rSDN + blood	X = -0.315, Y = 11.53	0.998

Fig. 3. RT-PCR validation for all DNA formulations in fresh mouse blood. Standard curves were obtained for all the DNA formulations tested both with and without the addition of fresh mouse blood (DNA (*diamonds*) DNA+blood (*squares*) rPLL/DNA (*triangles*) rPLL/DNA+blood (×) rSDN (*) rSDN+blood (*circles*)). Linear fit parameters are shown in the table.

the purpose of this study. To facilitate quantitative dissociation of the polyplexes, it was necessary to modify the composition of the lysis buffers by adding a reducing agent and a polyanion. Therefore, the modified lysis buffer was formulated by supplementing tris-hydroxypropyl-phosphine to reduce disulfide bonds in the rSDN and poly-L-aspartic acid to dissociate the polyplexes. RT-PCR calibration curve for luciferase plasmid DNA exhibits excellent fit with R^2= 0.9914 in a concentration range 0.2 ng ml^{-1} to 20 μg ml^{-1} (Fig. 2). The method was further validated by incubating the DNA formulations with mouse blood (Fig. 3). The RT-PCR calibration curves obtained for the different DNA formulations in blood show an excellent fit with $R^2 \geq 0.995$ in the same concentration range as above. In addition, the standard curves obtained in the presence of blood had almost identical slope and intercept as in the case of samples measured in the absence of blood (Fig. 3b). Considering that doses of intravenously (i.v.) administered DNA polyplexes in mice typically range from 1 to 100 μg per animal, the method provides sufficient sensitivity even at the lower concentration limit used. Assuming that 1 μg DNA per mouse equals initial blood concentration of ~500 ng ml^{-1}, the one-step RT-PCR method allows determining as low as 0.04% of the initially injected DNA dose, thus providing sufficient sensitivity for most PK studies. These results also indicate that the presence of blood does not negatively affect the quantification of the DNA in polyplexes and that the method is suitable for the quantification of intact functional DNA concentration in *in vivo* samples. The method was further validated in the presence of DNase to verify that the lysis buffer inhibits DNA degradation rapidly. The presence of DNase had no significant effect on the standard curve of naked DNA, confirming its reliability for PK analysis.

Pharmacokinetics of DNA and its Polyplexes

The main advantage of using RT-PCR in analyzing the PK of DNA is that the method determines the active

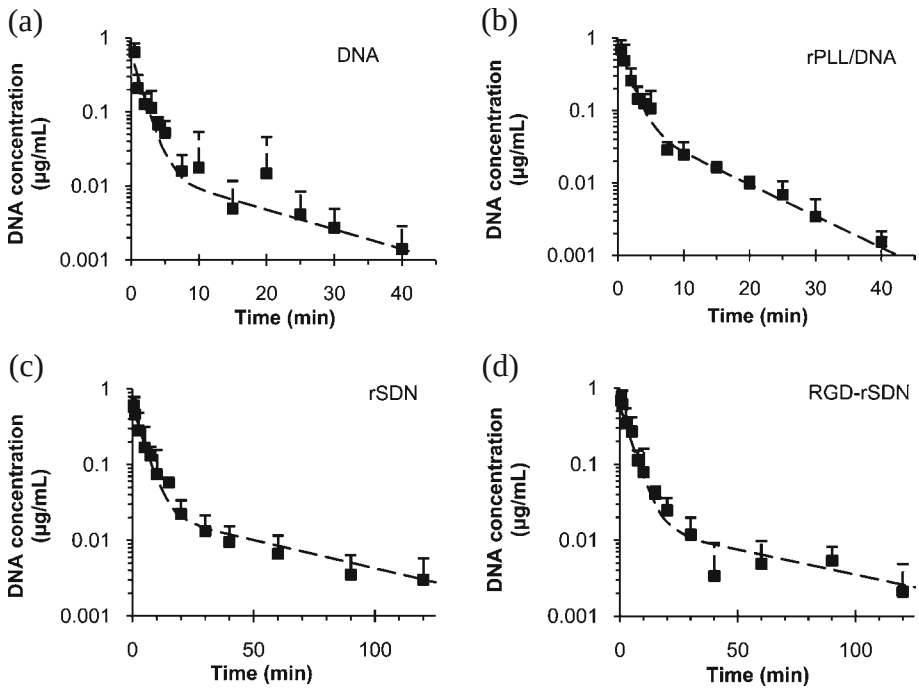


Fig. 4. Plasma clearance of the DNA delivery vectors. Plasma clearance after i.v. injection of 1 μg DNA was determined for (**a**) naked DNA, (**b**) rPLL/DNA, (**c**) rSDN, (**d**) RGD-rSDN. Data were analyzed by PKAnalyst® and best fit is shown.

Table I. Parameters for Two-compartment Model and Non-compartment Model in PKAnalyst® Software

Two-compartment model	DNA	rPLL/DNA	rSDN	RGD-rSDN
A ($ng \cdot ml^{-1}$)	600.7	847.2	578.4	567.9
B ($ng \cdot ml^{-1}$)	16.2	70.6	23.8	16.0
A	0.718	0.7	0.25	0.23
B	0.061	0.1	0.017	0.015
K_0 ($ng \cdot ml^{-1} \cdot min^{-1}$)	0.55±0.12	0.47±0.04	0.16±0.02	0.17±0.05
K_{12} ($ng \cdot ml^{-1} \cdot min^{-1}$)	0.14±0.07	0.16±0.04	0.08±0.02	0.06±0.03
K_{21} ($ng \cdot ml^{-1} \cdot min^{-1}$)	0.079±0.03	0.14±0.04	0.026±0.007	0.021±0.002
$t_{\alpha 1/2}$(min)	0.97	1.02	2.8	3.0
$t_{\beta 1/2}$(min)	11.3	6.9	40.4	45.6
AUC ($ng \cdot ml^{-1} \cdot min^{-1}$)	1,102	1,951	3,699	3,501
C_0 ($ng \cdot ml^{-1}$)	602±152	917±70	602±40	584±163
Non-compartment model	DNA	rPLL/DNA	rSDN	RGD-rSDN
AUMC ($ng \cdot ml^{-1} \cdot min^{-1}$)	5,491	8,871	89,999	80,053
MRT (min)	5.0	4.5	24.3	22.9
V_d (ml)	1.66	1.09	1.66	1.71
CL ($ml \cdot min^{-1}$)	0.91	0.51	0.27	0.29

pharmacologic agent, i.e. intact functional DNA, unlike the alternative methods based on fluorescently or radioactively labeled DNA, which measure the label and not intact DNA. It was previously reported that stabilization of DNA polyplexes with HPMA copolymers can extend circulation half-life substantially (22). There are limited PK data available, however, for bioreducible DNA polyplexes stabilized with HPMA copolymers. Unlike non-bioreducible polyplexes, the bioreducible rSDN are potentially susceptible to disulfide reduction in the blood and subsequent destabilization. We have therefore investigated plasma clearance of four different formulations in C57/BL6 mice bearing subcutaneous B16F10 tumors: naked DNA, rPLL/DNA polyplex, rSDN, and RGD-rSDN (Fig. 4). The selected samples allowed us to investigate the effect of HPMA stabilization of bioreducible DNA polyplexes on their PK. B16F10 cells are known to exhibit high levels of RGD-binding integrins and thus were used to evaluate the effect of RGD targeting on the PK of rSDN and

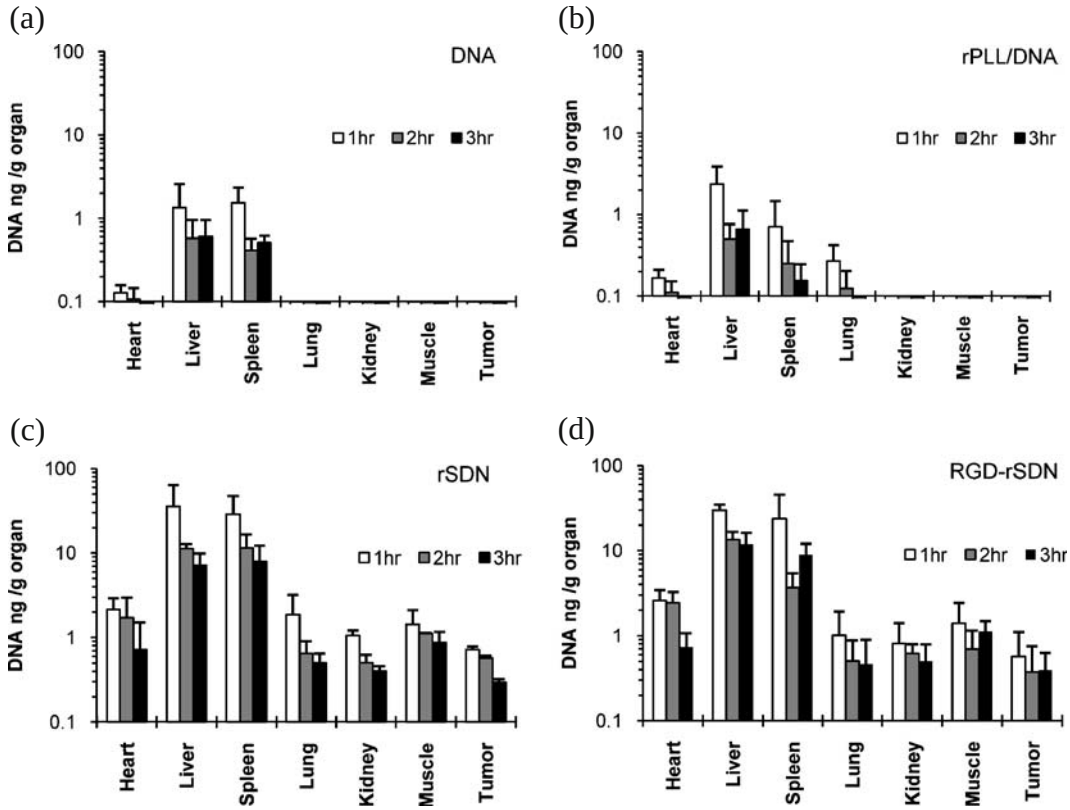


Fig. 5. Organ distribution of the DNA delivery vectors. Tissue distribution after i.v. injection of 1 μg DNA was determined in B16F10-bearing C57B6 mice for (**a**) naked DNA, (**b**) rPLL/DNA, (**c**) rSDN, (**d**) RGD-rSDN.

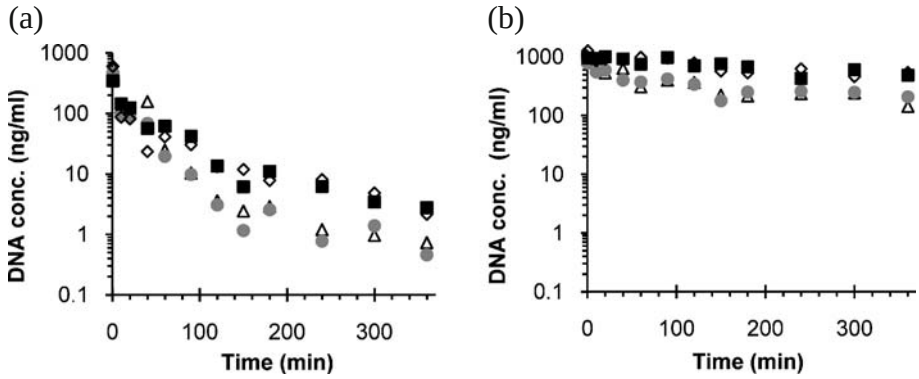


Fig. 6. DNA degradation in fresh whole mouse blood. Blood stabilized with (**a**) heparin and (**b**) sodium citrate: DNA (*triangles*), rPLL/DNA (*circles*), rSDN (*squares*) RGD-rSDN (*diamonds*).

RGD-rSDN. The clearance parameters were determined using two-compartment and non-compartment model in PKAnalyst® and are shown in Table I. The AUC analysis demonstrate that the HPMA-stabilized rSDN (3,699 ng·ml^{-1}·min^{-1}) and RGD-rSDN (3,501 ng·ml^{-1}·min^{-1}) have about 3.5 fold higher AUC than naked DNA (1,102 ng·ml^{-1}·min^{-1}) and about 2-fold higher AUC than parent rPLL/DNA polyplex 1951 ng·ml^{-1}·min^{-1}. At the same time, MRT increased 5-fold from 5 min, in case of naked DNA and polyplex, to about 25 min in case of rSDN and RGD-rSDN. According to the two-compartment model, naked DNA and polyplex have a very short $t_{\alpha 1/2}(\sim 1$ min) , while rSDN and RGD-rSDN show about 3-fold longer half-life (3 min). Simple rPLL/DNA polyplexes have the lowest apparent volume of distribution (V_d) suggesting interactions of the positively charged polyplexes with blood proteins that affected distribution to other organs and tissues. The distribution time $t_{\alpha 1/2}$ is only around 1 min for DNA and rPLL/DNA, which is far less than distribution times of typical small molecule drugs. The MRT of the plasmid DNA determined by RT-PCR was also was in agreement with published results that used radiolabeled DNA (19). HPMA-stabilized PLL/DNA polyplexes exhibited prolonged half-life of about 90 min in Balb/c mice (22). Bioreducible rPLL/DNA polyplexes stabilized polyplexes showed a lower but still significantly extended half-life in Balb/c mice using radiolabeled DNA to follow the PK (7). When analyzed by RT-PCR in this study, DNA in the rSDN was eliminated rapidly from the plasma. This suggests that a combination of clearance of the particles and DNA degradation due to a partial or complete polyplex disassembly in the circulation takes place. It is possible that partially reduced rSDN retain improved circulation over rPLL/DNA polyplexes but cannot provide sufficient DNA protection against nuclease degradation.

Organ Distribution of Intact DNA in Tumor-bearing Mice

As shown in Fig. 5a, no detectable amount of DNA was found after i.v. injection of naked DNA in most of the evaluated organs within 1 h of injection. A small amount, corresponding to less than 0.1%, of administered DNA was detected in the liver and spleen 1 h post-injection and the amount further declined at 2 and 3 h. Taken together with published data on PK of radiolabeled DNA, this observation clearly suggests not only hepatic and splenic clearance of circulating DNA but also rapid DNA degradation in the two organs and plasma. For example, more than 50% of administered radioactivity was observed in the liver 30 min post-injection of radiolabeled DNA (16, 25, 26). As shown in Fig. 5b, non-stabilized rPLL/DNA polyplexes provided very limited protection to DNA and similar amounts of intact DNA were found in the liver and spleen as in case of naked DNA. Unlike naked DNA, polyplexes mediated delivery of small amount of intact DNA into the lungs. rSDN and RGD-rSDN delivered significantly higher amount of intact DNA in all tissues compared with the naked DNA and polyplex. While only ~1 ng of intact DNA was recovered 1 h post-injection in case of naked DNA and polyplex, rSDN protection resulted in ~40 ng of intact DNA remaining. Specific uptake of rSDN and RGD-rSDN in the tumor was similar or lower to that observed in muscle, indicating no selective active or passive tumor targeting. The lack of enhanced delivery of intact DNA in the tumor is not surprising considering the fast clearance and it confirms the published observation that it requires at least several hours for targeted systems to accumulate in the tumor (27).

In Vitro Investigation of the Stability of Polyplexes

The *in vivo* data clearly suggested low stability of polyplexes, despite previous reports of improved PK and

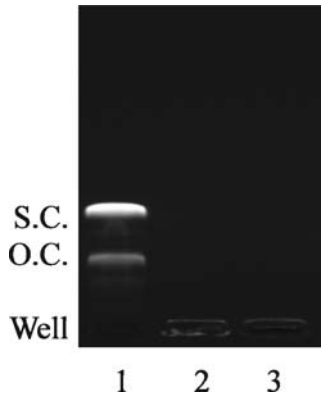


Fig. 7. DNA release by polyelectrolyte exchange with polyanion. The release of DNA in the presence of 1 mg ml^{-1} poly-L-aspartic acid: *1* rPLL/DNA polyplex; *2* rSDN; *3* RGD-rSDN. (*S.C.* supercoiled plasmid, *O.C.* open circular plasmid).

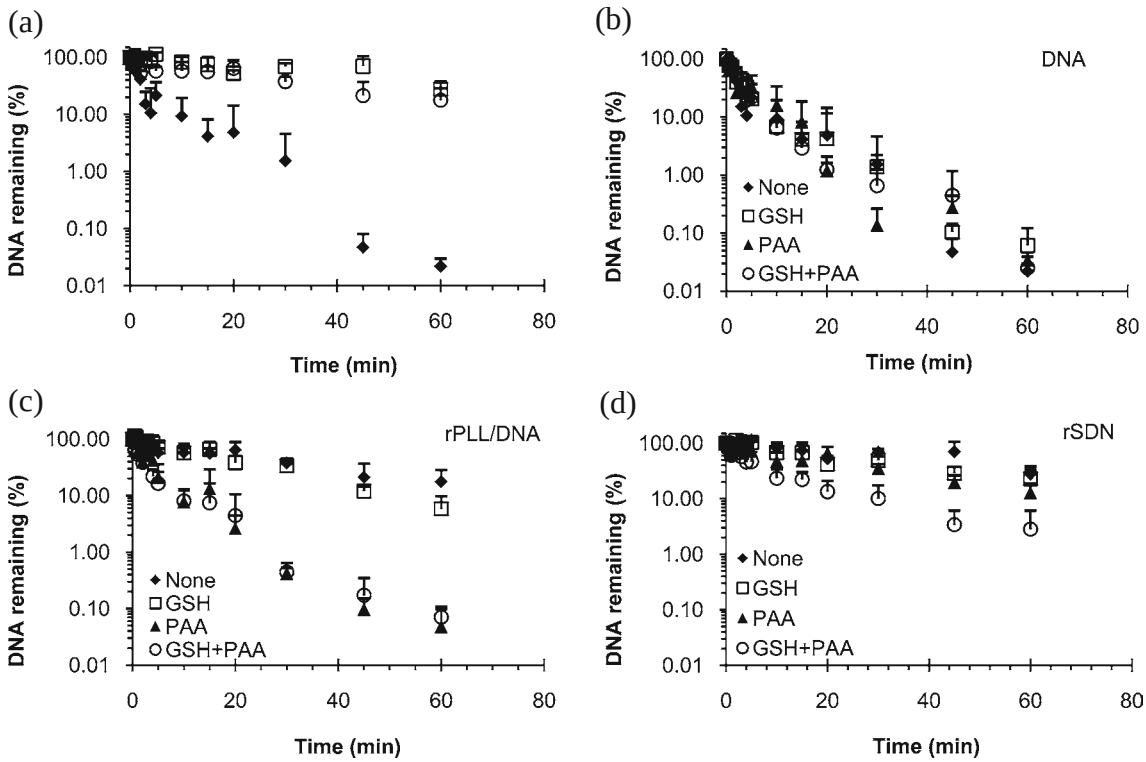


Fig. 8. Effect of glutathione and polyanion treatment on DNA degradation in the delivery vectors *in vitro*. (**a**) Degradation of naked DNA (*diamonds*), rPLL/DNA (*circles*), and rSDN (*squares*) in the presence of 10 U ml^{-1} DNase I. Different treatments (none, GSH, PAA, and GSH+PAA) were applied to naked DNA (**b**), rPLL/DNA (**c**), and rSDN (**d**). Amount of DNA remaining at different time points was quantified with RT-PCR.

stability of the stabilized polyplexes. Even though all our studies were conducted with much lower DNA doses (1 μg/mouse) than previous reports and it was established that the plasma clearance of the HPMA-stabilized polyplexes is dose-dependent (22), the apparent easy destabilization of rSDN warranted further investigation. Since the nuclease degradation appears to be a main reason for the poor performance, further investigation was focused on the stability of the formulations *in vitro*. We first set to study the enzymatic degradation of DNA in blood (Fig. 6). Since it was widely reported that blood contains significant levels of DNases (28, 29), it was reasonable to expect that fast degradation of DNA in blood will occur. Degradation of naked DNA and DNA in rPLL/DNA polyplex, rSDN, and RGD-rSDN was determined by mixing the formulations with freshly isolated heparinized mouse blood (Fig. 6a). More than 90% of DNA was degraded within 10 min in all the samples studied. Although rSDN and RGD-rSDN provided improved stability to DNA, the degradation was fast with half concentration eliminated within 2–3 min. The rate of DNA degradation was significantly reduced when incubated in citrate-treated blood (Fig. 6b). Sodium citrate acts as a non-specific inhibitor of DNases (30, 31), thus these data confirm the important contribution of DNA degradation to the observed pharmacokinetics of the polyplexes. Competitive binding of proteins present at high concentration in the blood could cause DNA release and explain the susceptibility of rPLL/DNA polyplexes to degradation. The stabilized polyplexes (rSDN) are resistant to such competitive reactions (Fig. 7) and rSDN need to

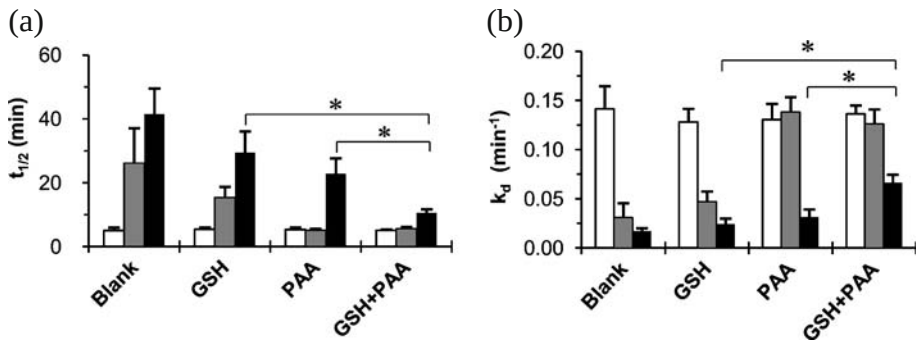


Fig. 9. Kinetic parameters of *in vitro* DNA degradation in the different formulations. Degradation half-life (**a**) and degradation rate constant (**b**) for DNA (*empty bars*), rPLL/DNA (*grey bars*), and rSDN (*black bars*). *$p<0.01$ ($n=4$).

undergo reduction of the disulfide bonds first, before they can be destabilized with negatively charged blood proteins. The observed degradation of DNA in rSDN therefore suggested that reducing activity of blood (32) may be sufficient to destabilize the polyplexes and that blood is thus potentially a major contributor to *in vivo* degradation of DNA in rSDN. Even though blood contains only relatively low concentrations of small molecule thiols like GSH (1–5 μM) and Cys (~10 μM), there is a large pool of protein thiols (~400 μM), mostly in serum albumin. Although the protein thiols typically have low reactivity, their involvement in thiol-disulfide reactions in polyplexes, especially when combined with anionic character of albumin, might be feasible.

Next, we quantified the relative importance of disulfide reduction and polyelectrolyte exchange on the stability of rSDN against nuclease degradation (Fig. 8). The experiments were conducted in a DNase buffer consisting of 10 U DNase I and degradation was followed by quantifying DNA with RT-PCR. The effect of reduction and polyelectrolyte exchange was determined by addition of PAA (0.5 mg ml^{-1}) and/or GSH (0.5 mM). While naked DNA was degraded rapidly in the DNase buffer alone, both rPLL/DNA and rSDN provided almost complete protection against nuclease degradation (Fig. 8a), confirming previous reports (34). As shown in Fig. 8b, addition of PAA and/or GSH had no significant effect on the rate of nuclease degradation of naked DNA. Fig. 8c shows the effect of PAA and GSH on DNA degradation in rPLL/DNA polyplexes. GSH alone is not able to fully destabilize the polyplexes but the presence of PAA alone is clearly sufficient to release DNA and make it susceptible to rapid degradation. Combination of GSH and PAA does not increase the degradation rate any further. In contrast, rSDN polyplexes provide good level of protection to DNA against nuclease degradation as shown in Fig. 8d. Unlike non-stabilized rPLL/DNA polyplexes, the combination of PAA and GSH resulted in the fastest rate of DNA degradation, confirming that resistance against polyelectrolyte exchange is important for polyplex stability. Importantly, even treatment with PAA alone resulted in significant extent of DNA degradation in rSDN, despite the fact that no detectable amount of free DNA was detected by agarose gel electrophoresis after PAA treatment (Fig. 7). This suggests that partial exposure of DNA in the polyplexes is sufficient to allow access of DNases and leads to degradation. It is therefore conceivable that the stabilized polyplexes retain their overall physical shape and properties even though the DNA they carry is partially degraded. This would explain the discrepancy between PK data obtained in this study using RT-PCR and previous reports based on the use of radiolabeled DNA, which could not distinguish partially cleaved DNA from intact DNA in the polyplexes. In order to better understand the combined effect of GSH and PAA on the susceptibility of polyplexes to DNase degradation, we calculated DNA degradation rate constants (k_d) and half-lives assuming first-order kinetics (Fig. 9). The analysis shows that k_d for rSDN in the presence of both GSH and PAA differs significantly from k_d in the presence of either GSH or PAA. The results also show that for rSDN $k_{d(GSH+PAA)} > (k_{d(GSH)} + k_{d(PAA)})$ suggesting a synergistic effect of GSH and PAA in DNA degradation by DNases. While the conditions used in these experiments are not necessarily fully representative of *in vivo* conditions, the results nevertheless provide important clues about the effects of polyelectrolyte exchange and disulfide reduction on the *in vivo* PK behavior of rSDN.

CONCLUSIONS

RT-PCR was used to improve our understanding of the pharmacokinetics of bioreducible gene delivery vectors. The study shows that using a single-step lysis RT-PCR is highly sensitive and easily scalable method to analyze pharmacokinetics and biodistribution of intact DNA in the delivery vectors. The use of a single-step lysis buffer eliminates complications related to DNA extraction and purification from organs and tissues. RT-RCR is a useful tool to study plasmid DNA delivery vectors *in vivo* but also has the potential to be applied to siRNA delivery (34, 35). Our results confirmed that naked DNA and simple rPLL/DNA polyplexes have a short half life and MRT. HPMA-stabilized polyplexes can partially protect DNA from degradation *in vivo* by showing slightly extended half life. In addition, our results show that rSDN are susceptible to destabilization and DNA degradation in blood by a combined effect of disulfide reduction and exchange reactions.

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