In Vivo Gene Transfer Using Sulfhydryl Cross-Linked PEG-Peptide/Glycopeptide DNA Co-Condensates

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ABSTRACT: Recent interest in sulfhydryl cross-linked nonviral gene delivery systems, designed to trigger the intracellular release of DNA, has inspired studies to establish their utility *in vitro*. To determine if this concept can be extrapolated to *in vivo* gene delivery, sulfhydryl cross-linking peptides (dp 20), derivatized with either an N-glycan or polyethylene glycol (PEG), were used to generate sulfhydryl cross-linked gene formulations. The biodistribution, metabolism, cell-type targeting, and gene expression of sulfhydryl cross-linked PEG-peptide/glycopeptide DNA co-condensates were examined following i.v. dosing in mice. Optimal targeting to hepatocytes was achieved by condensing ^{125}I-DNA with an add-mixture of 10 mol % triantennary glycopeptide, 5 mol % PEG-peptide, and 85 mol % backbone peptide. Four backbone peptides were substituted into the formulation to examine the influence of peptide metabolism and disulfide bond strength on the rate of DNA metabolism and the level of gene expression *in vivo*. The half-life of DNA in liver was extended from 1 to 3 h using a backbone peptide composed of D-amino acids, whereas substituting penicillamine for cysteine failed to further increase the metabolic stability of DNA. Optimized gene delivery formulations transiently expressed secreted alkaline phosphatase in mouse serum for 12 days. The results suggest that disulfide bond reduction in liver hepatocytes proceeds rapidly, followed by peptide metabolism, ultimately limiting the metabolic half-life of sulfhydryl cross-linked DNA condensates *in vivo*. J Pharm Sci 92:1174–1185, 2003

Keywords: gene delivery; gene therapy; bioconjugates; DNA

INTRODUCTION

An optimal synthetic gene carrier must direct DNA to overcome a cascade of extracellular and intracellular barriers from the site of intravenous injection to the nucleus of target cells.[1–4] Cell type-specific targeting can be achieved by derivatizing peptides with a targeting ligand and a nonionic hydrophilic polymer, such as polyethylene glycol (PEG). The PEG performs the critical function of protecting DNA colloids from serum protein binding and recognition by the reticuloendothelial system (RES)[5] while the targeting ligand binds to cell surface receptors, facilitating endocytosis of the DNA condensate.

Bioconjugate carriers composed of peptides, polylysines, polyethylenimine, or dendrimers with covalently attached targeting ligand or PEG have been used to mediate gene delivery. Although peptide carriers are smaller and more homogenous, they are not sufficiently stable during circulation, resulting in premature dissociation of the DNA carrier complex, nonspecific targeting of DNA to the liver and rapid metabolism of

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naked DNA.[2,6–8] One approach to increasing DNA condensate stability provided by Anwer and colleagues was dipalmitoylation to form a short lipopeptide that, upon intravenous administration, mediated DNA uptake in the liver and reporter gene expression.[3]

Several cross-linking strategies have also been developed to stabilize peptide DNA condensates. Homobifunctional agents, such as dimethyl-3,3′-dithiobispropionimidate, were used to cross-link primary amines on the surface of peptide DNA condensates and thereby control their stability.[9–11] Glutaraldehyde was also used to cross-link glycopeptide/PEG-peptide DNA condensates by forming Schiffs bases between neighboring amines on the surface of DNA condensates.[2]

Recently, sulfhydryl cross-linking peptides and lipids were designed to undergo a template polymerization when bound ionically to DNA resulting in reversibly stabilized DNA condensates.[12–16] In principle, the reducing environment of the endosome and cytosol can trigger the release of DNA intracellularly, which may account for the improved gene transfer efficiency observed for these reagents *in vitro*.[14–16] Despite their demonstrated activity *in vitro*, none of the sulfhydryl cross-linked gene formulations under investigation have been evaluated for their ability to mediate gene expression *in vivo*. In the present study, we examine for the first time the *in vivo* properties of sulfhydryl cross-linked peptide DNA formulations by measuring their biodistribution, metabolic stability, and transient gene expression. The results indicate that despite their significant reductive stability measured *in vitro*, the intracellular reducing environment of the liver releases DNA prematurely resulting in rapid metabolism and significantly limiting the level of gene expression.

EXPERIMENTAL

Materials

Sodium ^{125}I was purchased from Dupont NEN (Boston, MA). Chloramine T, sodium metabisulfite, heparin, sodium dodecylsulfate (SDS), Sephadex G-25, D-mannitol, bovine serum albumin, Hepes, collagenase from *Clostridium histolyticum* type IV (lot number: 47H6865), carbonyl iron were purchased from Sigma (St. Louis, MO). Agarose was purchased from Gibco-BRL (Gaithersburg, MD). Methoxy-PEG-vinylsulfone 5000 Da was purchased from Fluka (Ronkonkoma, NY). SYBR-Gold was purchased from Molecular Probes, Inc. (Eugene, OR). Ketamine hydrochloride was purchased from Fort Dodge Laboratories (Fort Dodge, IA). Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) was purchased from Pierce (Rockford, IL). Xylazine hydrochloride was purchased from Miles Inc. (Shawnee Mission, KS). Silastic catheters (0.305 mm i.d. × 0.635 mm o.d.) were purchased from Baxter (Obetz, OH). ICR mice (30–35 g) were purchased from Harlan (Indianapolis, IN) and housed in cages located in a limited access area maintaining a 12-h light–dark cycle and controlled temperature (26–28°C). Ultrapure 100 and tip100, DNA purification columns were purchased from Qiagen (Santa Clarita, CA).

N-terminal Fmoc protected amino acids, 9-hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC), and diisopropylethylamine were obtained from Advanced ChemTech (Lexington, KY) and Bachem (King of Prussia, PA). Substituted Wang resin for peptide synthesis was obtained from Bachem (King of Prussia, PA). *N,N*-Dimethyformamide, trifluoracetic acid (TFA), acetic acid, acetonitrile, piperidine, and acetic anhydride were purchased from Fisher Scientific (Pittsburgh, PA). Peptide synthesis was performed on a computer interfaced Model 90 Advanced ChemTech solid phase peptide synthesizer (Lexington, KY). Peptide purification was performed using an analytical or a semipreparative (5 μm) C_{18} RP-HPLC column from Vydac (Hesperia, CA). Preparative HPLC was performed using a computer-interfaced HPLC and fraction collector from ISCO (Lincoln, NE). Electrospray mass spectrometry (ES-MS) was performed using a ThermoFinnigan LCQ mass spectrometer (San Jose, CA) interfaced with an analytical HPLC from Hitachi (San Jose, CA).

Radiolabeling Plasmid DNA

Endotoxin free plasmid DNA (5.1 kb), encoding a secreted form of human placental alkaline phosphatase (pSEAP) with the SV40 early promoter inserted upstream and the SV40 enhancer inserted downstream (Clontech, Palo Alto, CA), was purified from *Escherichia coli* using a Qiagen ultrapure column according to the manufacturers instructions. Plasmid DNA was radiolabeled with ^{125}I as described previously resulting in supercoiled and circular DNA with specific activity of 250 nCi/μg.[17]

Glycopeptide and PEG-Peptide Synthesis and Characterization

Sulfhydryl cross-linking PEG-peptides and glycopeptides were synthesized and characterized as reported previously.[18] Glycopeptide **1** is composed of a triantennary N-glycan attached through a thiol ether linkage to the side chain of the N-terminal Cys of Cys-Trp-Cys-$(Lys)_{15}$-Cys-Lys (Figure 1). PEG-peptide **2** is composed of a single PEG_{5000} chain attached through a vinylic sulfone linkage to the N-terminal Cys of Cys-Trp-$(Cys\text{-}Lys_3)_4$-Cys-Lys (Figure 1). Four backbone peptides (Figure 1, **3–6**) were used to alter the stability of sulfhydryl cross-linked DNA condensates. Peptides **3** and **4** were L- or D-Cys-Trp-$(Lys)_5$-Cys-$(Lys)_5$-Cys-$(Lys)_5$-Cys, respectively, while peptides **5** and **6** possessed L- or D-Pen-Lys-Trp-$(Lys)_7$-Pen-$(Lys)_8$-Pen, where Pen is β,β-dimethyl Cys. L-Cys peptide **3** and D-Cys peptide **4** were synthesized as described previously.[12] L-Pen peptide **5** and D-Pen peptide **6** were synthesized using standard Fmoc procedures with 9-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) double couplings followed by N-capping with acetic anhydride after each coupling to avoid deletion sequences. Trityl (Trt) was used to protect the side-chain thiol of Pen, whereas Lys and Trp were protected with tertbutoxycarbonyl (Boc). Following synthesis, peptides were cleaved from the resin with simultaneous side-chain deprotection with TFA/EDT/water (95:2.5:2.5 v/v/v) for 2 h at room temperature. A low coupling yield when attaching Pen to Trp necessitated changing the sequence of the final three residues on L-Pen peptide **5** and D-Pen peptide **6** from Pen-Trp-Lys into Pen-Lys-Trp. Likewise, to improve synthetic yields, the number of Pen residues in **5** and **6** were decreased from four to three. The chemical identity of Pen peptides **5** and **6** were verified by the LC-MS and LC-MS/MS.

Peptides were purified to homogeneity on RP-HPLC by injecting 1 μmol onto a Vydac C_{18} semipreparative column (2×25 cm) eluted at 10 mL/min with 0.1% TFA and a gradient of acetonitrile (5 to 25% over 30 min) while monitoring tryptophan absorbance at 280 nm. The major peak eluting at 24 min was collected and pooled from multiple runs, lyophilized, and stored dry at −20°C. Purified peptides were reconstituted in 0.1% TFA (degassed with nitrogen) and quantified by tryptophan absorbance ($\varepsilon_{280} = 5600\ M^{-1}\ cm^{-1}$) to determine the isolated yield was typically 20%.

Purified peptides were characterized by LC-MS and LC-MS/MS by injecting 10 nmol onto a Vydac C_{18} analytical column (0.47×25 cm) eluted at 1 mL/min with 0.1 v/v % acetic acid containing 0.02 v/v % TFA and an acetonitrile gradient of 1 to 25% over 30 min. The RP-HPLC eluent was directly infused into the electrospray ionization source of a ThermoFinnigan LCQ mass spectrometer and mass spectral data was obtained in the positive mode.[19,20]

Formulation of Cross-linked Glycopeptide and PEG-Peptide DNA Co-condensates

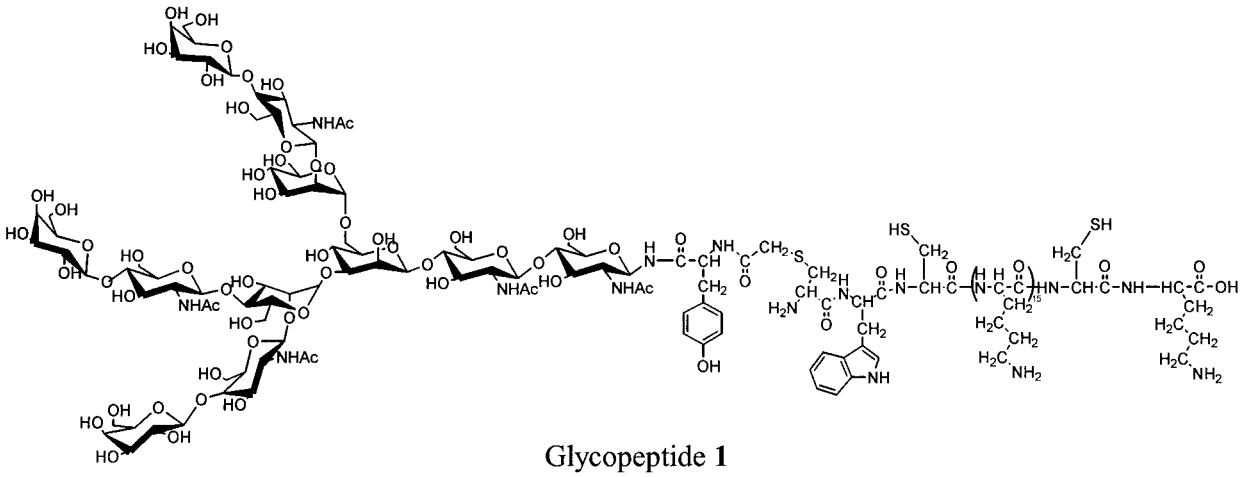
DNA co-condensates were prepared by add-mixing a backbone peptide (85 mol %) with glycopeptide **1** (10 mol %) and PEG-peptide **2** (5 mol %) in HBM (5 mM Hepes, 0.27 M mannitol, pH 7.4). pSEAP (37.5 μg in 375 μL) was combined with either 7.5 or 15 nmol of peptide add-mixture in 375 μL of HBM while vortexing. DNA co-condensates, prepared at either 0.2 or 0.4 nmol of peptide per μg of DNA corresponding to a charge ratio (NH_4^+/PO_4^-) of 1:1 or 2:1, formed instantly and cross-linked over 2 h but were allowed to equilibrate overnight at 4°C prior to analyzing particle size by quasi-elastic light scattering (QELS) and zeta potential on a Zeta-Plus (Brookhaven Instruments, Holtsville, NY).

Kinetics of Peptide Cross-linking

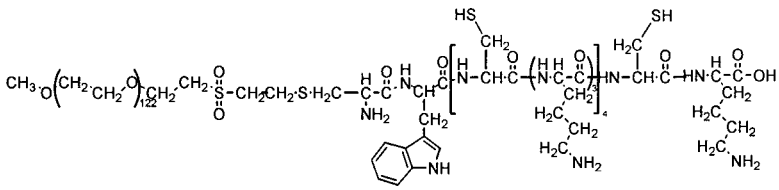
The kinetics of cross-linking within peptide DNA condensates were studied indirectly by monitoring the displacement of the SYBR-Gold intercalator dye from DNA condensates as a function of time.[12] Peptide DNA condensates were formed in 5 mM Hepes containing diluted (1:10,000) SYBR-Gold by combining 0.5 mL (20 μg) of DNA with 0.5 mL (8 nmol) of peptide. Immediately after mixing peptide and DNA, the fluorescence intensity (Ex 495 nm, Em 537 nm) was continuously monitored for 2 h.

Stability of Cross-linked DNA Condensates

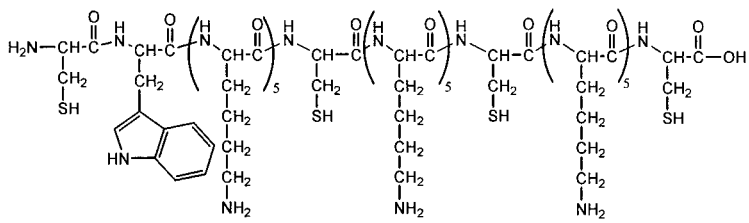
The stability of cross-linked DNA co-condensates was evaluated by adjusting 100 μL aliquots (5 μg DNA) to 0, 0.4, 0.8, 1.0, 1.5, 2.0, and 2.5 M sodium chloride (normalized to 300 μL with water) followed by sonication for 30 s with a 100 W Microson XL-2000 ultrasonic probe homogenizer (Kontes, Vineland, NJ) with a vibrational amplitude of 5 to fragment uncondensed DNA. Peptide



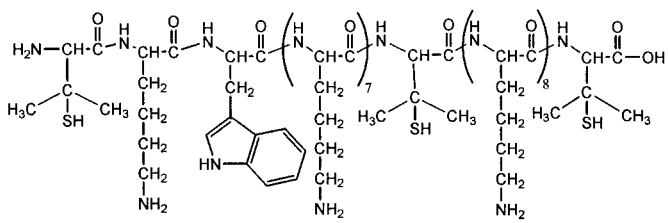
Glycopeptide **1**



PEG-peptide **2**



Peptide **3** or **4**



Peptide **5** or **6**

Figure 1. Structure of glycopeptide **1**, PEG-peptide **2**, and peptides **3**–**6**. The chemical structure of each bioconjuate carrier used is illustrated. L-Cys peptide **3** and L-Pen peptide **5** are composed of all L-amino acids, whereas D-Cys peptide **4** and D-Pen peptide **6** are composed of all D-amino acids.

DNA co-condensates (0.5 μg per well) were digested with 30 μg of trypsin in 3 μL of Tris buffer (50 mM Tris HCl, 7.5 mM $CaCl_2$, pH 7.5) for 12 h at 37°C in 1 M sodium chloride, and then combined with 3 μL of loading dye prior to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

The reductive stability of cross-linked DNA co-condensates was studied by gel retardation as a function of increasing concentration of a reducing agent. Peptide DNA co-condensates prepared at 0.2 nmol of peptide per 0.5 μg of DNA per well were reacted for 1.5 h at a final TCEP concentration of 0, 1, 3, 5, 10, 15, or 20 mM in a total volume of 30 μL. Each sample was brought to 1 M sodium chloride and then combined with 3 μL of loading dye prior to electrophoresis on a 1% agarose gel with 0.05% SDS and TAE buffer (40 mM Tris acetate, 2 mM EDTA).

Biodistribution of ^{125}I-DNA Co-condensates

Mice were anesthetized and a single catheter was placed in the left jugular vein. ^{125}I-DNA (2.5 μg in 50 μL of HBM, 0.6 μCi) or ^{125}I-DNA condensates were dosed i.v. followed by vein ligation. After 5, 15, 30, 60, 120, 240, 360, and 480 min, mice were sacrificed by cervical dislocation and the major organs (liver, lung, spleen, stomach, kidney, heart, large intestine, and small intestine) were harvested, rinsed with saline, and weighed. The radioactivity in each organ was determined by direct γ-counting and expressed as the targeting efficiency, defined as the percent of the dose in the target organ.

Isolation of Hepatocytes and Kupffer Cells

Mice were dosed i.v. tail vein with 20 mg of carbonyl iron in 0.2 mL of saline. After 45 min, mice were anesthetized and a single catheter was inserted into the right jugular vein and used to dose ^{125}I-DNA co-condensates (2.5 μg DNA in 50 μL, 0.6 μCi in HBM). Following 30 min of biodistribution, the portal vein was cannulated and used to administer 0.2 mL heparin (100 U/mL) followed immediately by the perfusion buffers. The liver was first perfused at 5 mL/min for 2 min with oxygenated (95% oxygen, 5% carbon dioxide) preperfusion buffer (calcium and magnesium free Hepes solution, pH 7.45, 37°C), and then at 3 mL/min for an additional 3 min. The liver was digested by perfusion with oxygenated Seglen's Buffer (pH 7.45, 37°C) containing 0.058% (w/v) collagenase type IV at 3 mL/min for 16–20 min. At the start of the perfusion the vena cava and aorta were cut, and at the completion, the liver was excised and placed in a Petri dish (4°C) and cut into small pieces. Cells were dislodged and dispersed in ice-cold Hank's solution containing 10 mM Hepes, pH 7.45, 0.1% BSA and then incubated at 37°C for 20 min with shaking (30 rev/min). The dispersed cells were passaged through a 73-μm mesh filter then transferred to a 35-mL glass tube. The iron-filled Kupffer cells were attracted to the wall of the tube with a magnet while other cells were decanted off. The procedure was repeated three times and the Kupffer cells were combined and resuspended in 0.8 mL Hank's Hepes Buffer. The remaining cell suspension was centrifuged at $50 \times g$ for 1 min and the supernatant was discarded. The pelleted hepatocytes were washed twice with ice-cold Hank's–Hepes buffer followed by centrifuging at $50 \times g$ for 1 min. The hepatocytes were resuspended in 2 mL Hank's–Hepes buffer, and the cell number and viability were determined by the trypan blue exclusion method. The amount of radioactivity associated with each cell fraction was determined by γ-counting.

In Vivo Gene Expression

Four mice were dosed in the tail vein by infusing 1 mL over 10 min of HBM containing either 50 μg of plasmid DNA or 50 μg of a DNA formulation. Blood (30 μL) was collected via the tail vein from four mice at day 1, 3, 6, 9, 12, 15, 18, 21, 25, and 30 after dosing. The blood was allowed to clot at room temperature, centrifuged at $13{,}000 \times g$ for 15 min, and the serum that was collected was stored frozen at −20°C until assayed for SEAP.

The chemiluminescent SEAP assay (Clontech, Palo Alto, CA) was used as instructed by the manufacturer. Briefly, 5 μL of mouse serum was added to 5 μL of the dilution buffer provided by the kit. Standard curves were generated by diluting human placental alkaline phosphatase with blank mouse serum. Samples and standards were placed in 1.5 mL microcentrifuge tubes and heated for 30 min at 65°C to deactivate endogenous alkaline phosphatase. After cooling on ice for 5 min, the samples were brought to room temperature, and 10 μL of assay buffer was added followed by 10 μL of substrate diluted with chemiluminescent enhancer. After a 45-min incubation, luminescence was measured on a luminometer (Turner Designs, Sunnyvale, CA) by integrating for 5 s. Relative

light units were converted into SEAP concentration (ng/mL of serum) using a standard curve.

RESULTS

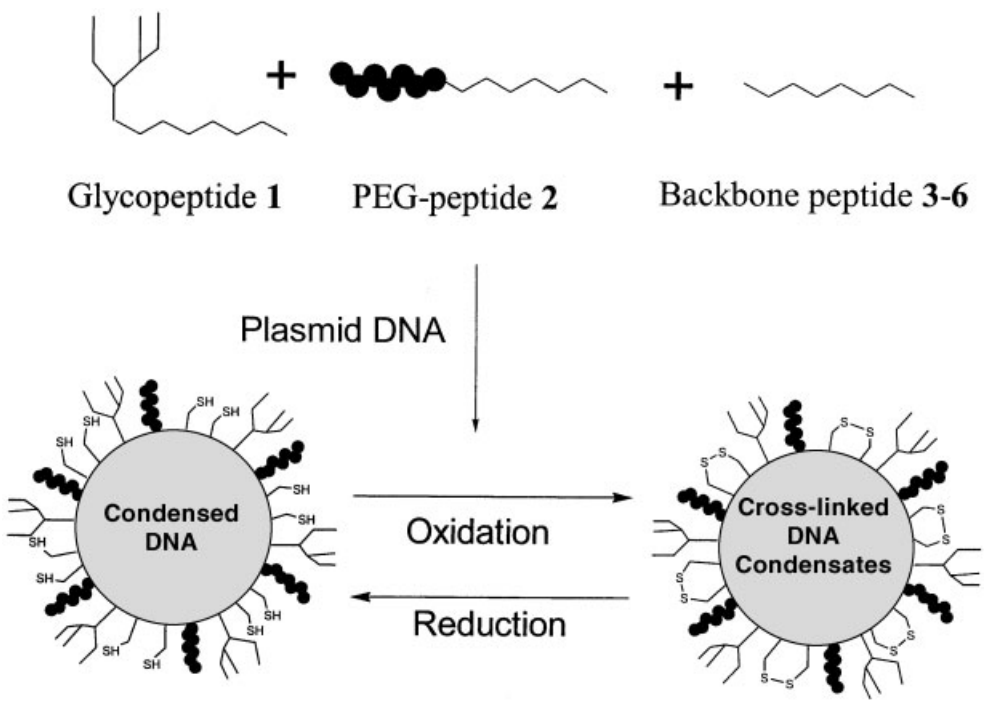
Characterization of Sulfhydryl Cross-linked DNA Co-condensate Formulations

To determine if sulfhydryl cross-linking peptides could achieve selective targeting and expression *in vivo*, gene formulations were developed that target the asialoglycoprotein receptor on hepatocytes (Scheme 1). Previous experiments demonstrated that as little as 2 mol % of a triantennary glycopeptide could selectively target glutaraldehyde cross-linked DNA condensates to hepatocytes while PEG-peptides were determined to be essential to block nonspecific recognition by RES cells.[2]

In addition to a sulfhydryl cross-linking glycopeptide and PEG-peptide (Figure 1), a third peptide component was substituted into the formulation to modulate the metabolic stability of the DNA. Four different backbone peptides possessing L- or D-amino acids containing either cysteine (Cys) (Figure 1, **3** and **4**) or penicillamine (Pen) (Figure 1, **5** and **6**) were substituted into the formulation. Because Pen disulfide bonds are nearly 60-fold more reductively stable than those formed with Cys,[21] comparison of DNA formulations prepared with 85 mol % of L-Cys peptide **3** or L-Pen peptide **5** with D-Cys peptide **4** or D-Pen peptide **6** were used to determine if disulfide bond strength or peptide dissociation and/or metabolism was the rate limiting step in the metabolism of DNA condensates.

Because the size and charge of DNA condensates strongly influence the *in vivo* performance of a gene delivery system, electronegative and electropositive DNA formulations were prepared at either a 1:1 or 2:1 charge ratio. QELS analysis established that regardless of charge, sulfhydryl cross-linked peptide DNA co-condensates formed small particles with a mean diameter of less than 100 nm (Table 1). Electronegative condensates had a zeta potential of approximately −14–16 mV, whereas electropositive condensates possessed a zeta potential of +14–21 mV (Table 1).

When bound to DNA, cross-linking peptides are less flexible, rendering intermolecular peptide



Scheme 1. Preparation of sulfhydryl cross-linked peptide DNA co-condensates. Peptide DNA co-condensates were formed by reaction of plasmid DNA with an add-mixture of glycopeptide **1**, PEG-peptide **2**, and one of either peptide **3–6**. The add-mixture ratio is systemically varied to optimize hepatocyte targeting and gene expression.

Table 1. *In Vitro* and *In Vivo* Properties of DNA Co-condensate Formulations

Dosage Form[a]	Particle Size[b] (nm)	Zeta Potential[c] (mV)	% Hepatocytes[d]	% Kupffer Cells[d]
A	69 ± 10	−13.0 ± 2.6	46 ± 6	54 ± 6
B	71 ± 7	−16.1 ± 1.8	65 ± 4	35 ± 4
C	64 ± 12	−15.0 ± 5.9	76 ± 1	24 ± 1
D	87 ± 13	14.4 ± 1.5	45 ± 3	55 ± 3
E	80 ± 9	18.1 ± 2.9	89 ± 2	11 ± 2
F	82 ± 9	19.6 ± 3.0	84 ± 4	16 ± 4
G	77 ± 8	21.5 ± 3.5	84 ± 2	16 ± 2
H	95 ± 10	19.8 ± 4.3	82 ± 2	18 ± 2

[a]Each formulation was prepared at a concentration of 50 μg/mL DNA and at a stoichiometry of 0.2 or 0.4 nmol of total peptides per μg of DNA corresponding to the charge ratio of 1:1 or 2:1.
A = glycopeptide **1**/PEG-peptide **2**/L-Cys peptide **3** (0/5/95 at a charge ratio of 1:1).
B = glycopeptide **1**/PEG-peptide **2**/L-Cys peptide **3** (5/5/90 at a charge ratio of 1:1).
C = glycopeptide **1**/PEG-peptide **2**/L-Cys peptide **3** (10/5/85 at a charge ratio of 1:1).
D = glycopeptide **1**/PEG-peptide **2**/D-Cys peptide **4** (0/15/85 at a charge ratio of 2:1).
E = glycopeptide **1**/PEG-peptide **2**/L-Cys peptide **3** (10/5/85 at a charge ratio of 2:1).
F = glycopeptide **1**/PEG-peptide **2**/D-Cys peptide **4** (10/5/85 at a charge ratio of 2:1).
G = glycopeptide **1**/PEG-peptide **2**/L-Pen peptide **5** (10/5/85 at a charge ratio of 2:1).
H = glycopeptide **1**/PEG-peptide **2**/D-Pen peptide **6** (10/5/85 at a charge ratio of 2:1).
[b]Particle size is reported as the mean diameter and standard deviation of five measurements.
[c]Zeta potential is reported as the mean and standard deviation of 10 measurements.
[d]Calculated as the percent of count per minute (cpm) in recovered hepatocytes (10^6) and Kupffer cells (10^5) over time.

cross-linking as the preferred route of oxidation.[12] A continuous fluorescence assay using SYBR-Gold as a DNA intercalator dye was used to determine the time course of interpeptide disulfide bond formation for peptides bound to DNA (Figure 2). At a charge ratio of 2:1, alkylated CWK_{18} formed DNA condensates instantly and produced a nearly constant SYBR-Gold fluorescence intensity over 2 h.[12] In contrast, the fluorescence intensity decreased over 30 min and reached an asymptote in 2 h when using formulation E or G (Table 1) composed of L-Cys peptide **3** and L-Pen peptide **5**, respectively (Figure 2). The kinetic decrease in SYBR-Gold fluorescence was interpreted as the time course of disulfide formation for peptides bound to DNA as has been demonstrated for other sulfhydryl cross-linking peptides, which upon polymerization more efficiently displace fluorophore due to higher binding affinity for DNA.[12]

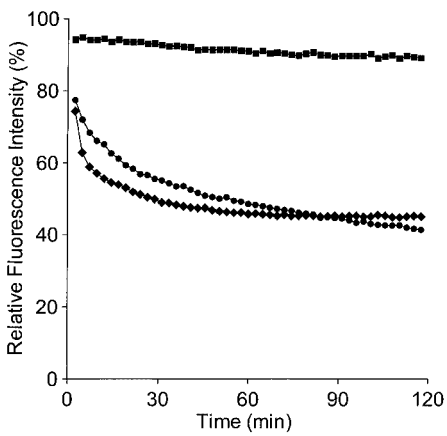


Figure 2. Kinetics of cross-linking DNA condensates. The fluorescence intensity of intercalated SYBR-Gold was continuously monitored over time during the formation of peptide DNA condensates prepared with alkylated CWK_{18} (■), and formulation E composed of L-Cys peptide **3** (◆) and formulation G composed of L-Pen peptide **5** (●). The results indicate a decrease in fluorescence over time for formulations composed of cross-linking peptides but not for alkylated CWK_{18}.

The stability of fully oxidized sulfhydryl cross-linked DNA condensates were evaluated as a function of increasing sodium chloride concentration to establish the nature of the disulfide cross-links with DNA condensates.[12,22,23] Previous studies established that 20 amino acid peptides possessing two terminal Cys residues linearly polymerized and formed DNA condensates that were stable up to 1 M sodium chloride, whereas peptides with three Cys residues formed DNA condensates that were stable in 2.5 M sodium chloride.[12] When peptide and DNA dissociate at a critical salt concentration, sonication leads to the fragmentation of OC and SC bands with the formation of a heterogeneous smear of smaller oligonucleotides.[12,22,23] The results presented in Figure 3A and B demonstrate that formulation C

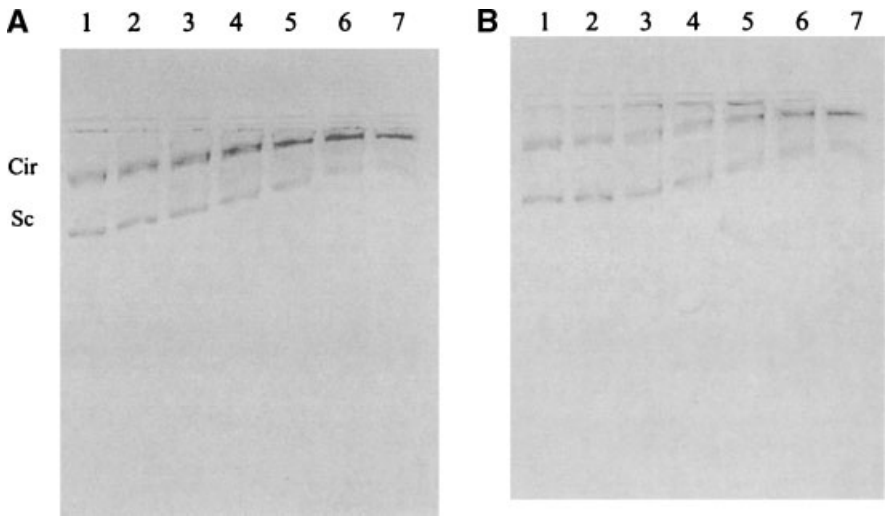


Figure 3. Shear stress stability of cross-linked peptide DNA co-condensates. The stability of cross-linked DNA condensates were measured by gel electrophoresis following a 30-s sonication in the prescence of increasing sodium chloride concentration. Lanes 1 through 7 represent 0, 0.4, 0.8, 1.0, 1.5, 2.0, and 2.5 M sodium chloride in the sonication solutions. Peptide DNA condensates were prepared using formulation C (A) or formulation E (B), both composed of L-Cys peptide **3** (Table 1). The results demonstrate the equivalent stability of electronegative and electropositive sulhydryl cross-linked DNA condensates.

and E, representing electronegative and electropositive formulations composed of L-Cys peptide **3**, protected DNA from fragmentation in 2.5 M sodium chloride. Apparently, upon binding to DNA, both linear polymerization and cross-linking occurred within peptide DNA condensates. The increase in salt concentration across the gel systematically retarded the migration of OC and SC DNA bands (assigned by comparison to standards), but no evidence of DNA fragmentation is observed (Figure 3).

The reductive stability of sulfhydryl cross-linked DNA condensates were compared by visual inspection of the DNA band intensity following reduction with increasing TCEP concentration. At a TCEP concentration of 1 mM or lower, formulation E, composed of L-Cys peptide **3**, prohibited the release of the DNA (Figure 4A, lanes 2 and 3), whereas at 3–20 mM, reduction of disulfide bonds caused the release of DNA from the condensates allowing recovery of bands on the gel (Figure 4A, lanes 4–8). By comparison, formulation G, composed of L-Pen peptide **5**, completely resisted reduction by TCEP up to 20 mM (Figure 4B, lanes 2–8) indicating that the Pen peptide has significantly higher reductive stability than the Cys peptide. Equivalent results were obtained for formulations F and H, possessing D-Cys or D-Pen peptides **4** and **6**, respectively (results not shown).

In Vivo Evaluation of Sulfhydryl Cross-linked DNA Co-condensates

To achieve maximal hepatocyte targeting selectivity, the stoichiometry of glycopeptide **1** was varied while monitoring targeting efficiency to hepatocytes. Previous studies have established the biodistribution equivalency of formulations possessing between 2.5 and 50 μg of DNA.[2] Formulation A (Table 1), which lacked a glycopeptide, distributed with 46% associated with hepatocytes and 54% with Kupffer cells, whereas formulation B, containing 5 mol % glycopeptide, improved the targeting specificity to 65% associated with hepatocytes. Increasing the glycopeptide to 10 mol % in formulation C increased hepatocyte targeting to 76%, whereas increasing the amount of PEG-peptide **2** did not improve the targeting selectivity. Comparison of formulation C and E established that electropositive formulations target hepatocytes with greater selectivity (Table 1). Biodistribution studies performed on electronegative DNA formulations A–C established the liver as the major target site at 15 min, resulting in a targeting efficiency of 55% with all other organs possessing $<10\%$ of the ^{125}I-DNA dose (Figure 5A). Analysis of the elimination rate of ^{125}I-DNA from the liver established a $t_{1/2}$ for DNA of approximately 1 h for formulation

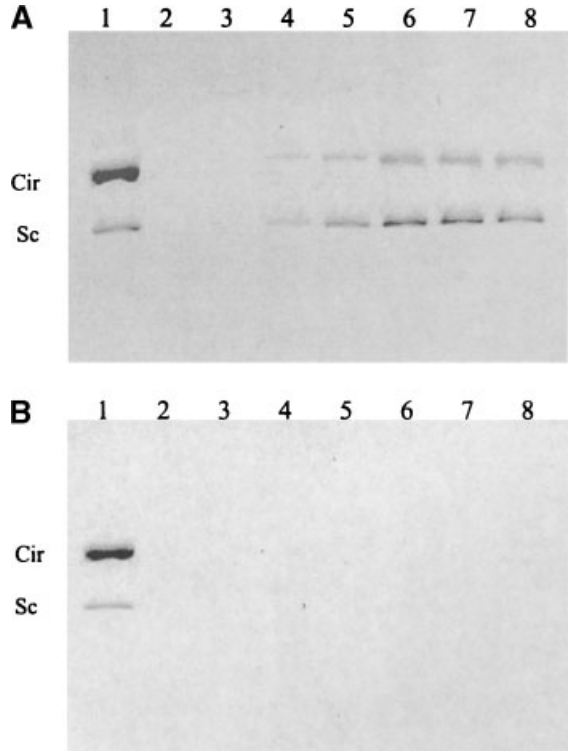


Figure 4. Reductive stability of sulfhydryl cross-linked peptide DNA co-condensates. The stability of cross-linked DNA co-condensates prepared with formulation E (A) and G (B) are compared by recovery of bands on gel electrophoresis. DNA formulations were treated with increasing concentrations of TCEP (lanes 2–8, 0, 1, 3, 5, 10, 15, and 20 mM) and compared to plasmid DNA treated with 20 mM TCEP (lane 1). The results indicate that formulation E, composed of L-Cys peptide **3**, released DNA whereas formulation G, composed of L-Pen peptide **5**, does not.

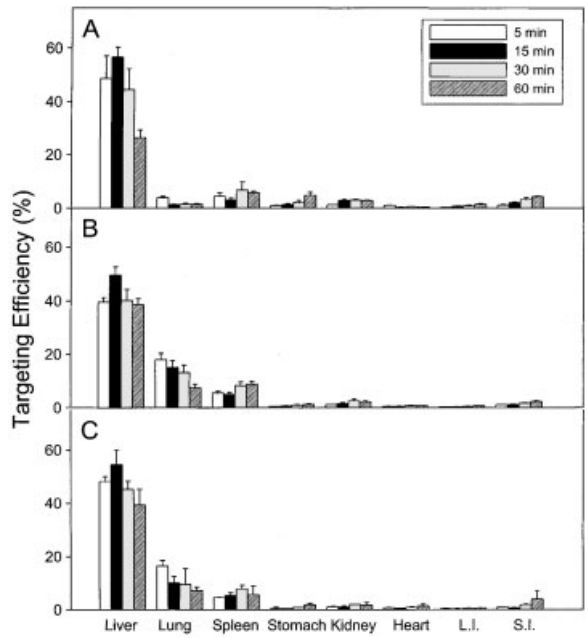


Figure 5. Biodistribution of ^{125}I-DNA condensates in mice. The targeting efficiency (percent of dose in the organ) was determined for ^{125}I-DNA formulations in the tissues at 5, 15, 30, and 60 min. (A–C) Displays the biodistribution profile of DNA formulations C, E, and F, respectively. The data represent the mean and standard deviation of triplicate mice.

A–C (Figure 6A–C), which is slightly longer than that of plasmid DNA that is rapidly eliminated from the liver with a $t_{1/2}$ of 0.6 h.[2] This result suggests that cross-linked electronegative DNA condensates only weakly protect DNA from metabolism in the liver. The finding that increasing the mol % of glycopeptide in formulation B and C improved selective targeting to hepatocytes supports the hypothesis that cross-linked condensates remain intact in circulation. We conclude that electronegative sulfhydryl cross-linked peptide DNA condensates undergo rapid metabolism in the liver perhaps due to endonuclease attack of exposed DNA.

Electropositive DNA condensates should provide greater protection from metabolism due to complete ion pairing of peptide with DNA. Biodistribution analysis of electropositive DNA co-condensates established the liver as the major target site at 15 min for all formulations, resulting in a targeting efficiency of approximately 50% (Figure 5B and C). A similar biodistribution profile was observed at 30 and 60 min for each formulation with proportional decreases in the liver targeting over time, without significant increases in the distribution to other tissues. The biodistribution time was extended to 8 h to determine the half-life of ^{125}I-DNA in the liver. At 2 h the ^{125}I-DNA remaining in the liver was threefold higher for formulations D–H relative to that obtained with uncross-linked DNA condensates.[2] As discussed above, otherwise identical peptide DNA condensates prepared at a charge ratio of 2:1 exhibited a longer liver half-life than those prepared at a charge ratio of 1:1 (Figure 6C versus E).

Substitution of peptides containing Pen for Cys (Figure 6E versus G or Figure 6F versus H) established that disulfide bond strength did not influence the metabolic half-life of DNA condensates in the liver. Alternatively, comparison of formulation E and F (Figure 6E versus F) or G and H (Figure 6G and H) established that replacement of L-amino acids with D-amino acids increased the

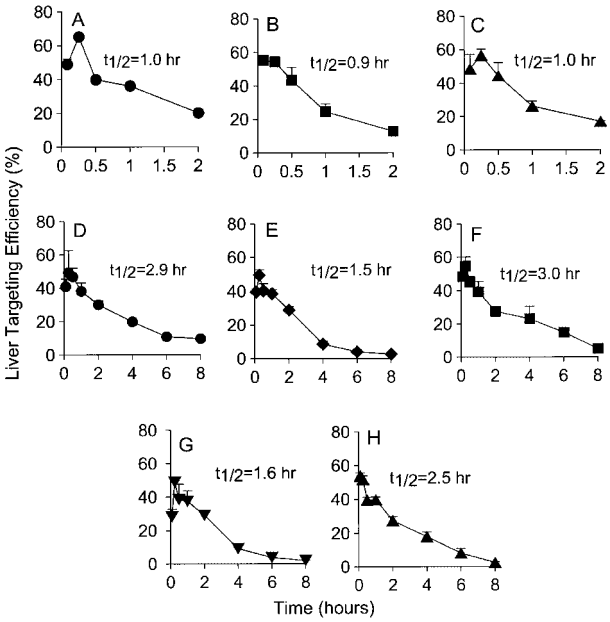


Figure 6. Liver biodistribution profile for ^{125}I-DNA formulations in mice. The time course of liver targeting and elimination of i.v. dosed ^{125}I-DNA formulations in mice are illustrated. (A–H) Illustrate the liver targeting efficiency (% of dose in liver) and the metabolic half-life for DNA formulations A–H (Table 1). Each result represents the mean and standard deviation of three independent analyses.

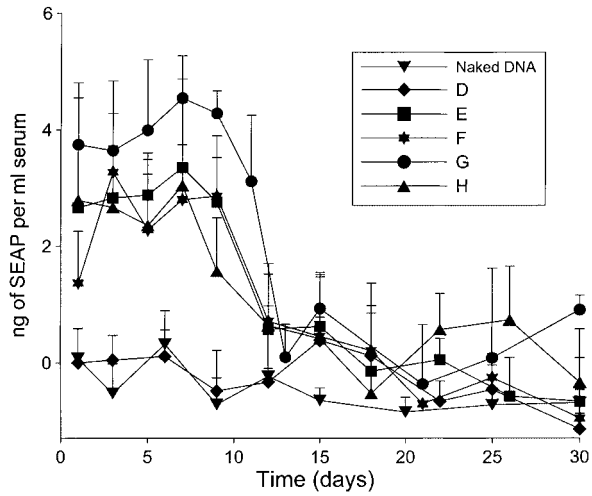


Figure 7. *In vivo* gene expression of sulfhydryl cross-linked DNA co-condensates. The 1-month gene expression profile of SEAP in mice is compared following a single 50-μg tail vein dose of DNA formulations D–H (Table 1) and naked plasmid DNA. Each formulation produced significantly higher levels of SEAP ($p < 0.05$) at day 7 relative to a control formulation (D). Each data point represents the mean and standard error for four mice.

liver half-life of DNA nearly twofold with 23% remaining in the liver at 4 h. These results suggest that peptide reduction of sulfhydryl cross-linked DNA co-condensate is most likely not the rate limiting step of DNA condensate metabolism in the liver. A rapid reduction of the peptides on DNA followed by a rate limiting dissociation and proteolytic cleavage is the most likely route of metabolism. This mechanism is supported by the finding that regardless of the reductive stability, sulfhydryl cross-linking peptides composed of D amino acids retard the rate of DNA metabolism *in vivo*.

Sulfhydryl cross-linked peptide DNA co-condensates were able to mediate SEAP expression *in vivo*. Analysis of the transient SEAP expression profile demonstrated a detectable gene expression at day 1, which peaked at day 7 at 3–5 ng/mL and returned to baseline levels by day 12 (Figure 7). Formulations E–H produced nearly equivalent transient gene expression profiles. Control formulation D, containing no targeting ligand, failed to produce gene expression, an identical result to that obtained when dosing naked plasmid DNA (Figure 7). Notably, the relatively short half-life of SEAP in blood (~17 h) suggests that the gene product detected in serum is the result of ongoing gene expression rather than persistent circulation of active protein in the serum.[24]

DISCUSSION

One function of polylysine and other polymers used as carriers for gene delivery is to protect DNA from premature metabolism.[6,23] The degree of metabolic protection afforded to DNA by polylysine is proportional to its molecular weight because longer polylysines bind to DNA with higher affinity and create more stable peptide DNA condensates.[6] An alternative strategy is to stabilize DNA formulations by cross-linking.[2] Sulfhydryl cross-linking peptides that form reversible interpeptide disulfide linkages[12,13] could offer an advantage over irreversible cross-linkers that limit the accessibility of the DNA for transcription and, hence, reduce gene expression.[22] Once internalized, the reducing environment could trigger disulfide bond reduction and intracellular release of DNA for gene expression.

Stable electronegative and electropositive peptide DNA condensates could be prepared because

sulfhydryl cross-linking is independent of peptide/DNA stoichiometry. At either charge ratio, electrophoretic analysis established that sulfhydryl cross-linked DNA co-condensates were stable against sonicative shear stress up to 2.5 M sodium chloride (Figure 3). The reductive stability of sulfhydryl cross-linked DNA condensates were also significantly greater when substituting Pen peptide for Cys peptide (Figure 4). To endow the delivery system with targeting specificity and simultaneously shield the surface charge of DNA condensates, peptides were conjugated to either a natural triantennary N-glycan ligand or PEG resulting in glycopeptide **1** and PEG-peptide **2**.

Electronegative formulations A–C formed small particles that avoided lung targeting (Figure 5A) more efficiently than electropositive particles, suggesting that a negative charge is advantageous in minimizing opsonization of DNA condensates in the blood to ensure good tissue dissemination after i.v dosing. An optimal formulation resulted in a higher specific uptake by hepatocytes (76%) than Kupffer cells (24%). However, the short half-life of DNA in the liver suggested that electronegative DNA condensates are more susceptible to serum nucleases.

Electropositive DNA formulations were more successful at mediating specific targeting to hepatocytes via the asialoglycoprotein receptor and achieving a longer liver half-life. Although formulations D–H were positively charged they avoided targeting to the lung as the primary site due to the use of a PEG-peptide, whereas without a PEG-peptide, electropositive DNA condensates have been shown to bind to serum proteins and are primarily trapped in the capillary beds of the lung.[2]

Formulations composed of D-amino acids formed condensates that were more stable in the liver relative to identical formulations possessing L-amino acids. This result is analogous to that reported when analyzing DNA formulations prepared with L- or D-polylysine.[25] What was unexpected was the nearly identical short liver half-life of DNA condensates prepared using Cys versus Pen containing peptides. Although L-Pen peptide **5** contained only three Pen residues, it formed DNA condensates that were significantly more reductively stable than peptide L-Cys peptide **3** DNA condensates possessing four Cys residues per peptide (Figure 4). This indicates that regardless of the strength of the disulfide bonds, reduction of the peptide proceeds faster than proteolysis to release DNA in lysosomes. When proteolysis is blocked by the use of D peptides, reduction of the disulfide bonds followed by dissociation of monomeric peptides is the proposed route of DNA release. This process is slower, accounting for the longer liver half-life of DNA, but was also not influenced by the nature of the disulfide bonds. These findings suggest that the concentration of endogenous thiols, such as glutathione, are sufficient to reduce even highly stabilized DNA condensates resulting in a relatively short liver half-life of 2.5 h.

The SEAP reporter gene was chosen to permit sensitive detection of serially sampled blood time points from individual mice.[26] *In vivo* expression of SEAP peaked at day 7 and lasted for 12 days with a level comparable to a previous study.[27] Comparison of the sulfhydryl cross-linked DNA condensates established an identical transient gene expression profile regardless of backbone peptide. In the absence of glycopeptide or carrier peptides, mice failed to produce measurable levels of SEAP. These results are consistent with previous studies that also determined a dependency on ligand to achieve alpha-1-antitrypsin gene expression, and found that a DNA liver half-life of less than 3 h only produced low levels of gene product.[2,28]

In conclusion, the results presented demonstrate that it is possible to use a combination of peptide conjugates to achieve cell-type specific gene delivery and transient gene expression in mouse liver by means of sulfhydryl cross-linking. These carriers offer the advantage of controlled synthesis and defined purity to allow strategic optimization to increase expression levels. Further studies will be needed to optimize these carriers to increase the metabolic half-life and the level of transient gene expression. This will likely involve the application of cross-linking strategies with transmembrane activity to assist endosomal escape.

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