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The Structure-Activity Relationship of PEGylated Polylysine Peptides as Scavenger Receptor Inhibitors for Non-Viral Gene Delivery

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Abstract

PEGylated polylysine peptides of the general structure PEG_{30kDa}-Cys-Trp-Lys (N =10 to 30) were used to form fully condensed plasmid DNA (pGL3) polyplexes at a ratio of 1 nmol of peptide per μg of DNA (ranging from N:P 3:1 to 10:1 depending on Lys repeat). Co-administration of 5 to 80 nmols of excess PEG-peptide with fully formed polyplexes inhibited the liver uptake of ^{125}I -pGL3-polyplexes. The percent inhibition was dependent on the PEG-peptide dose and was saturable, consistent with inhibition of scavenger receptors. The scavenger receptor inhibition potency of PEG-peptides was dependent on the length of the Lys repeat, which increased ten-fold when comparing PEG_{30kDa}-Cys-Trp-Lys₁₀ (IC₅₀ of 20.2 μM) with PEG_{30kDa}-Cys-Trp-Lys₂₅ (IC₅₀ of 2.1 μM). We hypothesize that PEG-peptides inhibit scavenger receptors by spontaneously forming small 40 to 60 nm albumin nanoparticles that bind to and saturate the receptor. Scavenger receptor inhibition delayed the metabolism of pGL3-polyplexes resulting in efficient gene expression in liver hepatocytes following delayed hydrodynamic dosing. PEG-peptides represent a new class of scavenger inhibitors that will likely have broad utility in blocking unwanted liver uptake and metabolism of a variety of nanoparticles.

Graphical Abstract

Keywords

scavenger receptor inhibition; liver biodistribution; polyplex; nonviral gene delivery; polyethylene glycol peptide

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Introduction

The non-parenchymal cells of the liver are a major barrier to successful non-viral and viral gene delivery¹. Kupffer cells and fenestrated endothelial cells bind and engulf plasmid DNA, nanoparticles, viruses, anionic liposomes, and DNA polyplexes resulting in metabolism of the majority of the dose²⁻⁹.

Foreign circulating viruses and plasmid DNA bind to scavenger receptor A1 (SR-A1)¹⁰ displayed on the surface of both Kupffer cells and fenestrated endothelial cells. SR-A1 is the prototypic and most abundant member of a larger family of scavenger receptors that bind diverse anionic ligands such as oxidized low density lipoprotein (LDL) and high density lipoprotein (HDL), acetylated LDL, maleylated and malondialdehyde bovine serum albumin (BSA), fucoidan, dextran sulfate, polyinosinic acid (Poly-I), poly guanine (Poly-G) and gram-positive and negative bacteria¹¹. SR-A1 is a homo-trimeric membrane spanning receptor with a short N-terminal 5 amino acid cytoplasmic tail, an 163 amino acid extracellular stalk composed of a coiled coil helix combined with a 72 amino acid collagen-like repeat, and a 110 amino acid C-terminal Cys-rich ligand binding domain¹¹. A cluster of four essential Lys residues located in the outer most C-terminal portion of the receptor line the binding pocket for anionic ligands¹¹.

Adenovirus appears to be primarily taken up by SR-A1 on Kupffer cells⁹, whereas plasmid DNA is reportedly cleared by SR-A1 found on both Kupffer cells⁵ and liver fenestrated endothelial cells¹². SR-A1 has been shown to be saturable under dose-escalation of adenovirus¹³ or plasmid DNA^{5,12,14}. Inhibition of SR-A1 with Poly-I led to an increase in adenovirus transduction of hepatocytes^{15,16}. Similarly, inhibition of Kupffer cell SR-A1 with Poly-I effectively blocked liver uptake resulting in increased oncolytic measles virus transduction in solid tumor¹⁷.

SR-A1 and other scavenger receptors on Kupffer cells and liver fenestrated endothelial cells collaborate to recognize and phagocytose foreign polymers and particles followed by activation of the innate immune response with release of TNF- α into blood¹⁸. The i.v. administration of plasmid DNA and lipoplexes¹⁹ activates the innate immune response. Intra-biliary administration of plasmid DNA, chitosan-DNA and PEI-DNA also significantly increases (10-fold) serum concentration of TNF- α ²⁰. The realization that adenovirus is primarily taken up by Kupffer cells via scavenger receptors, and that higher doses of the adenovirus (10¹²) leads to Kupffer cell death and the release of serum lactate dehydrogenase¹³, prompted the use of pre-administered Poly-I to inhibit adenovirus accumulation in Kupffer cells and to improve hepatocyte transfection⁹. While Poly-I is reportedly not toxic to mice, co-administration of Poly-I with adenovirus decreases the lethal threshold for adenovirus in mice⁹. Thereby, the use of Poly-I to inhibit scavenger receptor mediated uptake in liver is not a viable approach to improve the efficiency of gene delivery in humans.

We previously reported that PEGylated polyacridine peptides form stable polyplexes that protect DNA from metabolism when dosed i.v. in mice²¹. Detailed biodistribution studies

revealed that stable polyplexes are taken up by liver scavenger receptors²². The liver uptake was saturable under dose escalation of polyplex or PEG-peptide, consistent with the saturation of scavenger receptors. Saturation of scavenger receptors significantly decreased the percent of dose captured by the liver, increased the polyplex pharmacokinetic half-life, and increased the metabolic half-life of the polyplexes associated with liver²². Scavenger receptor inhibition allowed a 1 µg polyplex dose to circulate and remain fully transfection competent for 12 hours in mice²².

The present study examined the structure-activity relationship of PEGylated polylysine peptides as scavenger receptor inhibitors in mice. A 10-fold increase in scavenger receptor inhibitory potency was revealed when comparing PEGylated polylysine peptides with Lys repeat of 10 versus 25 residues. PEGylated polylysine peptides bind to albumin to form nanoparticles that are proposed to compete for and saturate scavenger receptor binding. The ability to delay the metabolism of polyplexes by dosing excess PEG-peptide to inhibit scavenger receptors results in significant increases in non-viral gene transfer efficiency in the liver.

Experimental Section

Unsubstituted Wang resin and diisopropylcarbodiimide (DIC) were from Advanced ChemTech (Louisville, KY, USA). Fmoc-protected amino acids, N-hydroxybenzotriazole (HOBt), O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), and N, N-dimethylformamide (DMF) were from AAPPTec (Louisville, KY, USA). Trifluoroacetic acid (TFA) and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Diisopropylethylamine, piperidine and acetic anhydride were purchased from Sigma Chemical Co. (St Louis, MO, USA). Maleimide-mPEG_{30kDa} was obtained from Laysan Bio (Arab, AL, USA). D-Luciferin and luciferase from *Photinus pyralis* were obtained from Roche Applied Science (Indianapolis, IN, USA). pGL3 control vector, a 5.3-kbp luciferase plasmid containing a SV40 promoter and enhancer, was obtained from Promega (Madison, WI, USA). pGL3 was amplified in a DH5α strain of *Escherichia coli* and purified using a Qiagen giga prep according to the manufacturer's instructions.

Synthesis and Characterization of PEGylated Polylysine Peptides

Peptides were prepared by solid phase peptide synthesis on a 30-µmol scale using an APEX 396 synthesizer (Advanced ChemTech, Louisville, KY, USA) with standard Fmoc procedures as described previously²¹. The Cys residue on Cys-Trp-Lys_N was PEGylated by reacting 2 µmols of peptide with 2.4 µmol of mPEG_{30kDa}-maleimide in 4 ml of 100 mM ammonium acetate buffer pH 7 for 2 h at room temperature. PEGylated polylysine peptides were purified by semi-preparative RP-HPLC eluted at 10 ml/min with 0.1 v/v % TFA with a 20–50 v/v % acetonitrile gradient over 30 min. The major peak was collected and pooled from multiple runs and concentrated by rotary evaporation, lyophilized and stored at –20°C until further use. The trifluoroacetate counter ion was exchanged by two freeze drying cycles with 1 v/v % acetic acid. PEG-peptides were then reconstituted in water and quantified by absorbance (tryptophan $\epsilon_{280\text{ nm}} = 5600\text{ M}^{-1}\text{cm}^{-1}$) to determine isolated yield (Table 1). PEG-peptides were prepared for ¹H NMR by dissolving 250 nmols in 0.5 ml of D₂O

(99.96%) containing 0.01% acetone as the internal standard. ^1H -NMR spectra were acquired on a Varian 600 MHz spectrometer and used to integrate the ratio of PEG ethylene protons to Lys side chain ϵ -methylene protons to determine the molecular weight of the PEG-peptide, as previously reported²³.

Formulation and Characterization of PEG-Peptide Polyplexes

The charge ratio resulting in fully formed polyplexes was determined by band shift assay. PEG-peptides (PEG_{30kDa}-Cys-Trp-Lys_N, where N= 10, 15, 20, 25, or 30) were combined with 1 μg of pGL3 at an N:P charge ratio ranging from 1–10, corresponding to a PEG-peptide to DNA ratio of 0.1–1 nmol per μg of DNA. Samples were electrophoresed on a 1% agarose gel containing 0.05% ethidium bromide for 2 hours at 70 V, then imaged using UVP BioSpectrum Imaging Systems and Vision Works LS software (UVP, Upland, CA, USA).

Particle size and zeta potential analyses were performed by preparing fully condensed pGL3 polyplexes at a concentration of 30 $\mu\text{g}/\text{ml}$ in 1.6 ml of 5 mM HEPES pH 7.5 at a constant stoichiometry of 1 nmol of PEG-peptide per 1 μg of pGL3 (N:P ranging from 3:1 to 10:1 depending on PEG-peptide Lys repeat). The particle size was measured by quasi-elastic light scattering (QELS) at a scatter angle of 90° on a Brookhaven Zetaplus particle sizer (Brookhaven Instruments Corporation, Holtzville, NY, USA). Intensity averaged multi-modal distribution analysis was used to determine the mean particle diameter and population width followed by zeta potential analysis determined as the mean of 10 measurements. The zeta potential of PEG-peptide polyplexes were also determined in 5 mg/ml bovine serum albumin (BSA) in 5 mM HEPES pH 7.5. PEG-peptide albumin nanoparticles were prepared by adding 80 nmol of PEG-peptide to 5 mg of BSA in 1 ml of 5 mM HEPES pH 7.5, followed by 30 min incubation prior to particle size analysis to determine the mean diameter and population width.

Biodistribution of PEG-Peptide DNA Polyplexes

^{125}I -pGL3 was prepared as previously reported²⁴. ^{125}I -pGL3 polyplexes were prepared by combined 1 μg (0.6 μCi) of pGL3 with 0, 5, 10, 20, 40, or 80 nmols of PEG-peptide in 100 μL of HBM (5 mM HEPES, 0.27 M mannitol, pH 7.4) to form 1 μg of pGL3 PEG-peptide polyplex containing an increasing excess of unbound PEG-peptide. ^{125}I -pGL3 polyplexes containing excess PEG-peptide were dosed in triplicate mice via the tail vein. At a biodistribution time of 5 min, mice were anesthetized by 100 μL intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then sacrificed by cervical dislocation. The major organs (liver, lung, spleen, stomach, kidney, heart, small intestine and large intestine) were harvested, rinsed with saline, and the radioactivity in each organ was determined by direct γ -counting and expressed as the percentage of dose in the organ.

Alternatively, the kinetics of biodistribution to the liver over 1 hour was monitored in mice that were anesthetized by administering a 200 μL i.p. dose of 100 mg/kg ketamine and 10 mg/kg xylazine, then placed in a supine position. A gamma probe (Ludlum model 44-3, 5.1 x 17.8 cm) was partially shielded with lead tape resulting in a 5 mm aperture that was positioned at a distance of 1 mm above the liver. Mice were dosed i.v. tail vein with 100 μL of ^{125}I -pGL3 (0.6 μCi) or ^{125}I -pGL3 polyplex with or without excess PEG-peptide followed

immediately by direct gamma counting of counts-per-minute (cpm) acquired continuously at a 1 min interval using a computer-interfaced gamma counting rate meter (Ludlum model 2200).

HD-Stimulated Gene Expression in the Liver

Direct hydrodynamic dosing (HD) was performed as described previously²⁵ by dosing 1.8 ml of saline (20 g mouse) containing 1 µg of pGL3 under high pressure via syringe within 5 seconds via the tail vein to achieve liver specific gene expression. To determine the circulatory stability of polyplexes, a recently described HD-stimulation protocol was used^{21,22,26,27}. The primary dose of pGL3 polyplex was administered via the tail vein in 100 µl followed, after a circulatory time, by a second dose of 1.8 ml of saline (20 g mouse) administered hydrodynamically under high pressure via syringe within 5 seconds. The circulatory time between primary and secondary dose in the present study was 1 or 2 hours.

HD-stimulation was performed by tail vein dosing triplicate mice with 1 µg of pGL3 prepared with 10, 40, or 80 nmol of PEG-peptide in 100 µl of HBM. At circulatory times of 1 or 2 hours, an HD-stimulatory dose of normal saline (9 wt/vol %) was administered in 5 sec via the tail vein. At 24 hours post HD-stimulation, mice were imaged for bioluminescence (BLI) on an IVIS Imaging 200 series (Xenogen, Hopkins, MA, USA) as previously described²¹. BLI results were analyzed for statistical significance by calculating the base 10 logarithm of each luminescence measurement. The log transformed data was analyzed by ordinary one-way analysis of variance (ANOVA) using GraphPad Prism version 6.02 (GraphPad Software, La Jolla, CA, USA), with Dunnet's Multiple Comparisons Test.

Results

PEG-Peptide Synthesis

A panel of polylysine PEG-peptides was prepared to determine the structural features needed to inhibit scavenger receptors mediated uptake of polyplexes by the liver (Table 1). Peptides of the general structure of Cys-Trp-Lys_N (N= 10, 15, 20, 25, or 30 Lys residue repeats) were prepared by solid phase peptide synthesis and purified by RP-HPLC resulting in isolated yields ranging from 19–33% (Table 1). LC-ESI-MS characterization established close agreement between the observed and calculated mass of the peptides (Table 1). PEGylation of the Cys residue with maleimide-PEG_{30kDa} afforded the PEG-peptides in 75–88% isolated yield. ¹H-NMR verified the structure of PEG-peptides by integration of protons at 3.62 ppm (PEG) relative to 2.92 ppm signal (Lys ε CH₂). The relative integrated peak area allowed calculation of PEG average molecular weight. The PEG molecular weight was combined with the precise peptide mass determined by ESI-MS to derive the average molecular weight for each PEG-peptide (Table 1)²³.

Particle Size and Zeta Potential Analysis of PEG-Peptide Polyplexes

Agarose gel electrophoresis was used to determine the PEG-peptide to DNA stoichiometry resulting in fully formed polyplexes. Complete DNA band shift were determined for each PEG-peptide at an N:P = 2, or 0.1–0.3 nmol per µg of DNA. To allow comparison across the range of PEG-peptides, polyplexes were formulated at a constant PEG-peptide to DNA

stoichiometry of 1 nmol of PEG_{30kDa}-Cys-Trp-Lys_N to 1 µg of pGL3 (N:P ranging from 3 to 10 depending on Lys repeat) to insure complete polyplex formation. The mean particle size determined for PEG-peptide polyplexes by DLS decreased from 200 to 150 nm when comparing PEG_{30kDa}-Cys-Trp-Lys₁₀ with PEG_{30kDa}-Cys-Trp-Lys₃₀ (Fig. 1A). The particle size did not change upon prolonged incubation. Zeta potential analysis revealed PEG-peptide polyplexes prepared with PEG_{30kDa}-Cys-Trp-Lys₁₀ resulted in a +3 mV charge, whereas PEG_{30kDa}-Cys-Trp-Lys₁₅ polyplexes resulted in +9 mV and PEG_{30kDa}-Cys-Trp-Lys₂₀, 25, and 30 resulted in +15 mV (Fig. 1C). Polyplex particle size and zeta potential were comparable when substituting 5 kDa PEG for 30 kDa PEG on Cys-Trp-Lys_N (where N 10, 15, 20, 25, and 30).

The particle size and zeta potential of PEG-peptide polyplexes were also measured in 5 mg/ml BSA to simulate interaction with blood proteins. Incubation of PEG-peptide polyplexes in 5 mg/ml BSA had a negligible effect on overall particle size (Fig. 1B). However, 5 mg/ml albumin decreased the charge on PEG-peptide polyplexes, resulting in a nearly equivalent charge of approximately +6 mV (Fig. 1D).

To simulate the interaction of free PEG-peptides (80 nmol) with albumin in the circulation, PEG-peptides were directly combined with 5 mg BSA in 1 ml of 5 mM HEPES pH 7.5 and analyzed for particle size. Albumin nanoparticles were detected by dynamic light scattering (Fig. 2). The particle size of albumin nanoparticles decreased as a function of increasing polylysine length. The average size was 60 nm for PEG_{30kDa}-Cys-Trp-Lys₁₀ which decreased to 43 nm for PEG_{30kDa}-Cys-Trp-Lys₃₀ (Fig. 2). Attempts to further characterize the albumin nanoparticles by sedimentation under centrifugation at 10,000 x g for 15 min were unsuccessful, likely due to the increased buoyancy resulting from association with PEG-peptide possessing a 30kDa PEG, or due to increase viscosity resulting from 5 mg/ml BSA.

Liver Biodistribution of PEG-Peptide Polyplexes

A 1 µg dose of ¹²⁵I-pGL3 (0.6 µCi) prepared with 0, 1, 5, 10, 20, 40, or 80 nmol of PEG-peptide (PEG-Cys-Trp-Lys_N, N= 10, 15, 20, 25, 30) was dosed i.v. tail vein in triplicate mice. The liver and other major organs were harvested at a biodistribution time of 5 min and directly gamma counted. The liver accumulated approximately 65% of the dose for each polyplex at a biodistribution time of 5 min, with all other organs accumulating less than 3% of dose. However, the percent of ¹²⁵I-DNA dose recovered in liver at a biodistribution time of 5 min decreased as a function of increasing PEG-peptide dose (Fig. 3). A plot of PEG-peptide dose (in µM, assuming a 2 ml blood volume in mice) versus the percent of ¹²⁵I-pGL3 polyplex dose recovered from liver at 5 min was fit by non-linear least squares regression using GraphPad Prism 6. The dose-response curve was used to determine the IC₅₀ for PEG-peptide inhibition of polyplex uptake by liver (Fig. 3). Polyplexes prepared with PEG_{30kDa}-Cys-Trp-Lys₁₀ produced an IC₅₀ of 20.1 µM. The addition of five Lys residues increased the inhibitor potency two-fold for PEG_{30kDa}-Cys-Trp-Lys₁₅, resulting in an IC₅₀ of 10.8 µM. Likewise, the addition of five Lys residues in PEG_{30kDa}-Cys-Trp-Lys₂₀ further increased the inhibitor potency resulting in an IC₅₀ of 5.5 µM (Fig. 3). The final addition of either five or ten Lys residues also doubled the inhibition potency for PEG_{30kDa}-Cys-Trp-

Lys₂₅ and 30, which were determined to be equipotent at inhibiting polyplex uptake in the liver, resulting in an IC₅₀ of 2.1 μ M (Fig. 3.)

The kinetics of biodistribution to liver was examined using a rate meter to continuously measure gamma counts per minute (cpm) in the liver following dosing ¹²⁵I-pGL3 and ¹²⁵I-pGL3-polyplexes i.v. tail vein to anesthetized mice. A 1 μ g (0.6 μ Ci) dose of ¹²⁵I-pGL3 resulted in a rapid rise to a peak count rate of 18000 cpm within 1 min followed by an increase in the metabolic half-life of ¹²⁵I-pGL3 from the liver with a half-life of 0.8 hours (Fig. 4). Administration of 1 μ g (0.6 μ Ci) of ¹²⁵I-pGL3 polyplex (1 nmol of PEG_{30kDa}-Cys-Trp-Lys₂₅) resulted in a delayed uptake into the liver reaching a maximum of 10000 cpm at 10 min followed by metabolic half-life of 1.2 hours (Fig. 4). In contrast, the administration of 1 μ g (0.6 μ Ci) of ¹²⁵I-pGL3 combined with 80 nmol of PEG_{30kDa}-Cys-Trp-Lys₂₅ resulted in rapid accumulation of only 5000 cpm in liver within 1 min which was eliminated slowly with a long metabolic half-life of 12 hours (Fig. 4). These results corroborate the inhibition results presented in figure 3, by indicating that an 80 nmol dose of PEG-peptide not only significantly blocks liver uptake, but that inhibition of liver uptake persists for at least 1 hour.

HD-Stimulated Gene Expression Using PEG-Peptide Polyplexes

A calibrated bioluminescence imaging assay was used to quantify luciferase expression in the liver of mice following hydrodynamic delivery of pGL3 or pGL3 polyplexes²⁸. A positive control performed by direct hydrodynamic dosing of 1 μ g of pGL3 resulted in luciferase expression in liver at 24 hours measured as 1×10^8 photons/sec/cm²/ster by quantitative bioluminescence imaging (BLI) (Fig. 5A, pGL3). A negative control performed by delivering 1 μ g of naked pGL3 in 100 μ l via the tail vein, followed by the delivery of a blank (no DNA) HD-stimulatory dose of saline (1.8 ml) at a 5 min circulatory time, resulted in no detectable luciferase expression in the liver due to rapid metabolism of pGL3 (Fig. 5A, Cont)²¹. Alternatively, a primary dose of 1 μ g of pGL3 polyplex containing 80 nmol excess of PEG_{30kDa}-Cys-Trp-Lys₁₅, followed HD-stimulation at 1 hour circulatory time, resulted in liver specific luciferase expression of 1×10^7 photons/sec/cm²/ser. This result established that PEG_{30kDa}-Cys-Trp-Lys₁₅ dosed in excess could partially protect pGL3 in the circulation for 1 hour. Substitution with PEG_{30kDa}-Cys-Trp-Lys₂₀ increased the expression 5-fold, whereas substitution with PEG_{30kDa}-Cys-Trp-Lys₂₅ increased expression an additional 5-fold, establishing it as equipotent with direct HD-dosing of 1 μ g of pGL3. These results established a direct correlation between PEG-peptide scavenger receptor inhibitor potency (Fig. 3) and the ability of excess PEG-peptides to protect pGL3 in the circulation for 1 hour (Fig. 5A).

To further establish a correlation between PEG-peptide scavenger receptor inhibition and the ability to protect pGL3 polyplex from metabolism, a dose-response study was performed using PEG_{30kDa}-Cys-Trp-Lys₂₅. Triplicate mice were administered a 100 μ l dose of 1 μ g of pGL3 polyplex combined with 10, 40 or 80 nmols of excess PEG_{30kDa}-Cys-Trp-Lys₂₅. Following the primary dose, a secondary HD-stimulatory dose of saline was administered at a 1 hour circulatory time and luciferase expression was measured at 24 hours. The results established that a 10 nmol excess dose of PEG-peptide resulted in no detectable luciferase

expression in liver, indicating 10 nmols was insufficient to delay polyplex metabolism during a 1 hour circulation time (Fig. 5B). A 40 nmol dose of PEG_{30kDa}-Cys-Trp-Lys₂₅ increased expression by 100-fold, establishing partial delay of polyplex metabolism at a 1 hour circulation time (Fig. 5B). Whereas an 80 nmol dose further improved expression by 15-fold, indicating that it fully protected the polyplex from metabolism for 1 hour (Fig 5B). However, polyplexes were not protected from metabolism at prolonged circulation times. Dosing 1 µg of pGL3 polyplex with an excess 80 nmols of PEG_{30kDa}-Cys-Trp-Lys₂₅ followed by HD-stimulation at a 2 hour circulatory time resulted in no detectable luciferase expression in the liver (not shown).

Discussion

The i.v. dosing of stable PEGylated DNA polyplexes results in rapid binding and uptake by scavenger receptors found on Kupffer cells and fenestrated endothelial cells in the liver leading to metabolism and deactivation of the encapsulated plasmid DNA²². This is coincident with previous observations that concluded scavenger receptors bind to i.v. dosed viruses, liposomes, plasmid DNA and nanoparticles, leading to their metabolism in Kupffer cells and fenestrated endothelial cells in the liver^{12,13,22,29}. Inhibition of scavenger receptor binding and uptake of polyplexes into these cells could slow metabolism and potentially improve the efficacy of non-viral delivery systems. We have recently reported that stable PEGylated polyplex uptake can be inhibited by dose-escalation of either polyplex or PEG-peptide²². The PEG-peptide used in that study possessed multiple Lys residues with interspaced Lys-Acr (acridine) residues to increase binding affinity to DNA. The polyacridine PEG-peptide formed albumin nanoparticles, inhibited polyplex uptake in the liver, increased the pharmacokinetic half-life of the polyplex, and extend the HD-stimulated gene expression²². Based on these earlier reported findings, we sought to determine if simpler PEG-peptides that lack Lys-Acr residues possess scavenger receptor inhibition properties and to determine the structural requirements of PEG-peptides that determine scavenger receptor inhibition potency.

Short synthetic polylysine peptides of the general structure PEG-Cys-Trp-Lys_N were developed nearly two decades ago in an effort to prepare well-characterized DNA binding peptides^{23,30}. In addition to homogeneity, synthetic polylysine peptides possessing an N-terminal Cys residue allowed uniform PEGylation without disrupting polylysine electrostatic binding to DNA. A subterminal Trp residue provided a hydrophobic chromophore facilitating both RP-HPLC purification and concentration determination. Cys-Trp-Lys_N (N= 8, 13, and 18) peptides form polyplexes at low N:P ratios (0.1–0.3), but were only weakly active at mediating in vitro transfection³⁰. PEGylation of the Cys residue decreased the polyplex charge and completely blocked the ability of PEG-peptide polyplexes to transfect cells in culture²³. PEG-peptide polyplexes were non-toxic to cells in culture and when dosed in mice^{8,31}. PEG_{5kDa}-Cys-Trp-Lys₁₈ formed polyplexes that resisted metabolism by serum DNase in vitro³². However, PEG-peptide polyplexes proved to be metabolically unstable when dosed i.v. in mice⁸, suggesting that short polylysine possessed insufficient DNA binding affinity to form stable polyplexes when dosed i.v.⁸. The present study challenges these earlier conclusions by demonstrating that PEG_{30kDa}-Cys-Trp-Lys_{10, 15, 20, 25, and 30}

polyplexes are metabolically stable in the circulation provided that excess PEG-peptide is administered to inhibit scavenger receptors in the liver.

Polyplexes are spontaneously formed when 1 μ g of pGL3 is combined with 1 nmol of PEG-Cys-Trp-Lys_N, with varying polylysine length (N) from 10–30 residues. This corresponds to a charge ratio (N:P) ranging from 3:1 to 10:1, which is already in excess over that needed to form polyplexes at N:P 2:1. The polyplex size decreased and charge increased with increasing Lys repeats, consistent with previous observations³⁰ (Fig. 1). PEGylation of the Cys residue with either PEG_{5kDa} or PEG_{30kDa} resulted in polyplexes of comparable size but with decreased charge when using PEG_{30kDa} due to charge masking, also consistent with previous observations²³. However, when dosed in excess the shorter PEG on PEG_{5kDa}-Cys-Trp-Lys₁₅, 20, 25, and 30 failed to inhibit polyplex uptake into the liver (not shown). We presume that low molecular weight PEG-peptides failed to inhibit polyplex uptake in the liver due to more rapid renal clearance. However, this hypothesis is in contrast to our previous report of a PEGylated polyacridine peptide possessing a 5 kDa PEG which was found to be a potent scavenger receptor inhibitor of polyplex uptake²². Therefore, there appears to be a more complicated relationship between PEG-peptide DNA binding affinity, polyplex charge, and PEG length that dictates which PEG-peptides will protect DNA in the circulation and exhibit scavenger receptor inhibition properties when dosed in excess. A more detailed investigation is needed to determine the molecular weight cutoff for PEG-peptides possessing PEG between 5 and 30 kDa to elucidate which function as scavenger receptor inhibitors.

Albumin binding to polyplexes influences their size and charge and alters biodistribution to the lung and liver²². Albumin causes aggregation of polyplexes that lack sufficient PEGylation, leading to biodistribution to the lung^{33–35}. In contrast, polyplexes possessing a sufficient density and length of PEG resist albumin induced aggregation and primarily biodistribute to the liver²⁶. Analysis of the particle size of PEG_{30kDa}-Cys-Trp-Lys_N polyplexes combined with 5 mg/ml albumin confirmed the lack of albumin-induced aggregation (Fig. 2).

Albumin binding to PEGylated polyplexes decreases their charge without causing aggregation²². Albumin binding has been shown to play a key role in polyplex recognition by scavenger receptors in the liver²². The addition of 5 mg/ml albumin decreased the charge of PEG_{30kDa}-Cys-Trp-Lys_N polyplexes from +15 down to +5 mV (Fig. 1C and D), consistent with previous charge reduction determined using stable PEGylated polyacridine peptide polyplexes²².

When directly combined with albumin, PEG_{30kDa}-Cys-Trp-Lys_N peptides spontaneously formed nanoparticles that are considerably smaller (40–60 nm) than polyplexes (150–200 nm) (Fig. 2). These results are consistent with the hypothesis that excess PEG_{30kDa}-Cys-Trp-Lys_N peptides bind to albumin in the circulation to form albumin nanoparticles that inhibit scavenger receptors²².

Dose-escalation of PEG_{30kDa}-Cys-Trp-Lys_N peptide resulted in a decrease in the amount of ¹²⁵I-pGL3 polyplex recovered in the liver at a 5 min biodistribution time (Fig. 3). The

decrease in liver uptake of polyplexes is most consistent with inhibition of scavenger receptors. The results fit ($r^2 = 0.99$) a saturation binding sigmoidal curve to allow calculation of an IC_{50} . The inhibition of liver uptake was directly correlated with polylysine chain length, with the potency of inhibition increased 10-fold when comparing PEG_{30kDa}-Cys-Trp-Lys₂₅ to PEG_{30kDa}-Cys-Trp-Lys₁₀ (Fig. 3). No correlation was found between inhibition potency and the size of albumin nanoparticles, as PEG_{30kDa}-Cys-Trp-Lys_N peptides of varying polylysine length each formed similar size albumin nanoparticles (Fig. 2). However, the inhibition potency in the liver is roughly correlated with polylysine binding affinity for DNA, which increases with length³⁰. Thereby, the observed increase in scavenger receptor inhibition potency when increasing the Lys repeat is likely related to the increased stability of the polyplex in the circulation. This hypothesis can be directly tested by demonstrating that PEG_{30kDa}-Cys-Trp-Lys₁₀ inhibits the liver uptake of stable PEGylated polyacridine peptide polyplexes or viruses.

A novel continuous monitoring rate-meter assay was used to measure the uptake and metabolism kinetics of ¹²⁵I-pGL3 and ¹²⁵I-pGL3-polyplex in the liver (Fig. 4). The rate metering assay was calibrated by dosing ¹²⁵I-pGL3. The rise in cps to 16,000 in 1 min is due to rapid uptake of 60% of the ¹²⁵I-pGL3 dose by the liver within 5 min due to scavenger receptor binding^{8,12,36}. The metabolism of naked ¹²⁵I-pGL3 in the liver results from digestion by endogenous DNase. This produces smaller ¹²⁵I-DNA fragments that appear in the blood and are eliminated by renal filtration⁸. The liver metabolic half-life has been previously determined from the percent of the ¹²⁵I-DNA dose remaining in liver at times ranging from 5 min to 2 hours following i.v. dosing⁸. Continuous rate metering allows rapid sampling of the percent of ¹²⁵I-DNA dose remaining in the liver of living mice over time. Fitting the results to a 1st order rate equation allows estimation of the liver metabolic half-life. The reported liver metabolic half-life for naked ¹²⁵I-pGL3 ($t_{1/2} = 0.6$ hour), based on gamma counting dissected livers⁸, compares favorably ($t_{1/2} = 0.8$ hours) with the metabolic half-life determined by continuous rate-metering.

The percent of polyplex dose taken up by the liver is dependent on the charge which is masked by covalent attachment of a dense layer of PEG²³. Compared to naked DNA, the maximal percent of polyplex dose taken up by liver was proximately 30% (Fig. 4). The metabolic half-life determined for naked DNA of 0.8 hours increased to 1.2 hours for PEG-peptide polyplex, due to the stability afforded by combining 1 nmol of PEG_{30kDa}-Cys-Trp-Lys₂₅ with 1 μ g of ¹²⁵I-pGL3. However, increasing the dose of PEG_{30kDa}-Cys-Trp-Lys₂₅ to 80 nmols further inhibited the uptake of polyplexes by the liver to approximately 16% (Fig. 4), and significantly increased the apparent metabolic half-life to 12 hours (Fig. 4). These results are consistent with inhibiting scavenger receptor mediated uptake of stable polyplexes with PEGylated polyacridine peptides²².

To demonstrate that pGL3 polyplexes remain fully transfection competent in the circulation, an HD-stimulation experiment was conducted. HD-stimulation is similar to direct hydrodynamic dosing (HD), with the exception that HD-stimulation separates the DNA dose in time from the hydrodynamic delivery of saline²⁷. Separating the doses create a DNA circulation time ranging from 5 min to several hours. Increasing the circulation time increases the stringency of the assay. Under the HD-stimulation procedure, the DNA dose is

only able to mediate luciferase expression if it remains intact in the circulation. A 1 µg dose of naked pGL3 serves as a negative control which undergoes complete loss of transfection competency when administered by HD-stimulation with a short 5 min circulation time as a result of rapid metabolism in blood and liver²⁷ (Fig. 5). In contrast, direct-HD of 1 µg of pGL3 serves as a positive control which bypasses both blood and liver metabolism of DNA resulting in maximal expression (Fig. 5). HD-stimulation is the most stringent in vivo gene transfer assay to test the ability of a polyplex to circulate and remain transfection competent²⁷. Thus far, only stable PEGylated polyacridine peptide polyplexes have possessed sufficient in vivo stability to mediate HD-stimulated expression, whereas all other polyplexes tested, which include PEI, chitosan, and lipoplexes prepared with lipofectamine, fail to demonstrate HD-stimulated expression²¹.

To test the circulatory stability of PEG-peptide polyplexes a HD-dose of saline was administered at 1 hour circulation time. This resulted in luciferase expression in the liver at 24 hours post HD-stimulation that increased as a function of increasing polylysine chain length, with PEG_{30kDa}-Cys-Trp-Lys₂₅ providing maximal expression (Fig. 5A). These results correlate liver uptake inhibition potency with polyplex stability, and the magnitude of HD-stimulated expression (Fig 3 and Fig 5A). However, compared to PEGylated polyacridine peptide polyplexes that remain fully transfection competent in the circulation for 12 hours²², the transfection competency of PEG_{30kDa}-Cys-Trp-Lys₂₅ polyplexes in the circulation was only preserved for up to 1 hour. This suggests that the ability to preserve DNA polyplex stability in the circulation is highly dependent on PEG-peptide binding affinity for DNA.

In conclusion, the results demonstrate that a PEG-peptide of optimal polylysine length (PEG_{30kDa}-Cys-Trp-Lys₂₅) protected a 1 µg dose of pGL3 in the circulation for an hour. The results support a proposed mechanism by which excess PEG-peptide forms albumin nanoparticles that inhibit scavenger receptors, thereby delaying polyplex metabolism. While this degree of metabolic protection is far less than that achievable with comparable PEGylated polyacridine peptides²² that intercalate DNA, these PEG-peptides are much easier to prepare because they lack Lys-Acr residues. These findings should help guide the development of non-viral delivery systems for the liver with improved efficiency. In addition, the PEG-peptides developed should have broad applications in blocking scavenger receptor mediated uptake of viruses and nanoparticles in the liver.

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Figure 1. Particle Size and Zeta Potential of PEG-Peptide pGL3 Polyplexes

Panel A and B compare the size of polyplexes prepared with PEG-peptides of differing polylysine ranging from 10–30 residues with and without 5 mg/ml BSA. Panels C and D illustrate the zeta potential of PEG-peptide polyplexes with and without 5 mg/ml BSA.

Figure 2. Particle Size of PEG-Peptide Albumin Nanoparticles

The mean size and standard deviation of albumin nanoparticles formed when combining PEG_{30kDa}-Cys-Trp-Lys_N (80 nmols) with BSA (5 mg/ml) is illustrated. The size decreases from 60–43 nm when the Lys repeat (N) increases from 10–30 residues.

Figure 3. Inhibition of Polyplex Uptake by the Liver

The percent of ^{125}I -pGL3 polyplex dose recovered from liver at a biodistribution time of 5 min is illustrated when administering 1–80 nmols of PEG_{30kDa}-Cys-Trp-Lys_N, where N is varying from 10–30 lysine residues, resulting in the IC_{50} values indicated.

Figure 4. Kinetics of PEG-Peptide Polyplex Biodistribution to the Liver

The biodistribution kinetics of 1 μg (0.6 μCi) of $^{125}\text{I-pGL3}$ to the liver was determined ($\blacktriangledown$), relative to the addition of 1 nmol ($\circ$) and 80 nmols ($\bullet$) of $\text{PEG}_{30\text{kDa}}\text{-Cys-Trp-Lys}_{25}$.

Figure 5. HD-Stimulated Gene Expression of PEG-Peptide Polyplexes

Panel A illustrates the magnitude of luciferase expression in liver following direct HD-delivery of 1 μ g of pGL3 followed by bioluminescence imaging (BLI) at 24 hours. I.V. delivery of 1 μ g of pGL3 in 50 μ l followed by HD-stimulation at 5 min and BLI at 24 hours results in no expression (Cont). Delivery of a primary dose of 1 μ g of pGL3 polyplex with 80 nmols of PEG_{30kDa}-Cys-Trp-Lys₁₅, 20 or 25 in 100 μ l followed by a secondary HD-stimulatory dose of saline at 1 hour and BLI at 24 hours establishes the level of expression depends on the length of the polylysine repeat. Panel B illustrates the results of varying the dose of PEG-Cys-Trp-Lys₂₅ from 10–80 nmols at a constant dose of 1 μ g pGL3 polyplex followed by HD-stimulation at 1 hour and BLI at 24 hours.

Table 1

PEGylated Polylysine Peptides.

Polylysine Peptide^a	% Yield	Mass (Calc./Obs.)
Cys-Trp-Lys ₁₀	33.4	1589.1/1589.0
Cys-Trp-Lys ₁₅	32.2	2229.9/2230.2
Cys-Trp-Lys ₂₀	28.8	2870.8/2870.6
Cys-Trp-Lys ₂₅	19.2	3511.7/3511.2
Cys-Trp-Lys ₃₀	18.9	4152.5/4152.3
PEG Peptide^b	% Yield	Mass (Calc./Obs.)
PEG _{30kDa} -Cys-Trp-Lys ₁₀	80.2	31589/31717
PEG _{30kDa} -Cys-Trp-Lys ₁₅	88.5	32230/32839
PEG _{30kDa} -Cys-Trp-Lys ₂₀	79.7	32871/33320
PEG _{30kDa} -Cys-Trp-Lys ₂₅	74.7	33512/34957
PEG _{30kDa} -Cys-Trp-Lys ₃₀	86.5	34152/36655

^aDetermined by ESI mass spectrometry^bDetermined by ¹H-NMR spectroscopy.