INTRODUCTION

The development of highly efficient nonviral gene delivery systems that mediate expression in animals following intravenous (i.v.) dosing remains a significant challenge. Delivery systems that are optimized in vitro often fail to mediate expression in vivo due to a combination of aberrant pharmacokinetics or biodistribution, inadequate intracellular trafficking or DNA release or the inability to penetrate the nucleus.

To systematically overcome the barriers associated with nonviral gene delivery to the liver, we have adopted a strategy of administering a 1 μg dose of pGL3 polyplex (in 50 μl) via the tail vein of mice. Following a delay time of 5 min to 9 h, a stimulatory hydrodynamic (HD) dose (1.8–2.25 ml depending on body weight) of saline is delivered via the tail vein and the resulting luciferase expression in the liver is measured by quantitative bioluminescence imaging (BLI) at 24 h. This approach circumvents optimization of parameters toward efficient in vitro gene transfer, by instead allowing direct optimization of parameters key to successful in vivo gene delivery. To be fully transfection competent by HD-stimulated delivery, DNA polyplexes must be sufficiently stable in the circulation to survive DNAse metabolism, must avoid protein binding and biodistribution to the lung and must release DNA intracellularly following HD stimulation.

Polyethylene glycol (PEG)ylated glycoproteins were the first gene delivery systems that produced measurable luciferase expression following HD stimulation. However, the level of expression was very low (10^5–10^9 photons sec⁻¹ cm⁻² ser⁻¹) and was only detectable at an escalated 5 μg dose of pGL3 polyplex and at a short HD-stimulation delay time of 5 min post DNA delivery. Without applying HD stimulation, these delivery systems fail to produce measurable luciferase expression in the liver.

To overcome the very limited efficacy of gene delivery systems to produce HD-stimulated expression, we developed PEGylated polyacridine peptides. Modification of the ε-amino of Fmoc-Lys with acridine (Acr) affords an amino acid that is incorporated into a peptide during solid phase synthesis. We reported that increasing the number of Acr residues from 2 to 6 greatly increases the binding affinity of polyacridine peptides for DNA. The identity of a spacing amino acid separating Acr residues also greatly influences DNA-binding affinity with Lys > Arg > Leu > Glu. Even with a time delay of up to 1 h post DNA delivery, (Acr-Lys)₆-Cys-PEG₅kDa pGL3 (1 μg) polyplexes mediated luciferase expression equivalent in magnitude to direct-HD dosing of 1 μg of pGL3.

Based on these encouraging results, we explored the relationship between the number of Lys residues spacing Acr residues within a PEGylated polyacridine peptide and its effects on magnitude (10^5–10^10 photons sec⁻¹ cm⁻² ser⁻¹). Although in vitro transfer of naked plasmid DNA fails to mediate significant levels of gene expression, likewise tail vein administration of 1–5 μg of pGL3 in 50 μl, followed by a 5-min delay before an HD-stimulatory dose of saline, results in no detectable luciferase expression, due to the rapid metabolism of DNA in the blood.

Polyethylene glycol (PEG)ylated polycationic peptides bind to plasmid DNA with high affinity to form unique polyplexes that possess a long circulatory half-life and are hydrodynamically (HD)-stimulated to produce efficient gene expression in the liver of mice. We previously demonstrated that acridine-modified lysine (Acr) in (Acr-Lys)₆-Cys-PEG₅kDa stabilizes a 1 μg pGL3 dose for up to 1 h in the circulation, resulting in HD-stimulated (saline only) gene expression in the liver, equivalent in magnitude to direct-HD dosing of 1 μg of pGL3. In this study, we report that increasing the spacing of Acr with either four or five Lys residues markedly increases the stability of PEGylated polyacridine peptide polyplexes in the circulation allowing maximal HD-stimulated expression for up to 5 h post DNA administration. Co-administration of a decoy dose of 9 μg of non-expressing DNA polyplex with 1 μg of pGL3 polyplex further extended the HD-stimulated expression to 9 h. This structure–activity relationship study defines the PEGylated polyacridine peptide requirements for maintaining fully transfection competent plasmid DNA in the circulation for 5 h and provides an understanding as to why polyplexes or lipoplexes prepared with polyethylenimine, chitosan or Lipofectamine are inactive within 5 min following intravenous dosing.

**Keywords:** gene delivery; peptide; pharmacokinetics; liver; hydrodynamics

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**High-affinity PEGylated polyacridine peptide polyplexes mediate potent in vivo gene expression**

K Kizzire, S Khargharia and KG Rice

Polyethylene glycol (PEG)ylated polyacridine peptides bind to plasmid DNA with high affinity to form unique polyplexes that possess a long circulatory half-life and are hydrodynamically (HD)-stimulated to produce efficient gene expression in the liver of mice. We previously demonstrated that acridine-modified lysine (Acr) in (Acr-Lys)₆-Cys-PEG₅kDa stabilizes a 1 μg pGL3 dose for up to 1 h in the circulation, resulting in HD-stimulated (saline only) gene expression in the liver, equivalent in magnitude to direct-HD dosing of 1 μg of pGL3. In this study, we report that increasing the spacing of Acr with either four or five Lys residues markedly increases the stability of PEGylated polyacridine peptide polyplexes in the circulation allowing maximal HD-stimulated expression for up to 5 h post DNA administration. Co-administration of a decoy dose of 9 μg of non-expressing DNA polyplex with 1 μg of pGL3 polyplex further extended the HD-stimulated expression to 9 h. This structure–activity relationship study defines the PEGylated polyacridine peptide requirements for maintaining fully transfection competent plasmid DNA in the circulation for 5 h and provides an understanding as to why polyplexes or lipoplexes prepared with polyethylenimine, chitosan or Lipofectamine are inactive within 5 min following intravenous dosing.

**Keywords:** gene delivery; peptide; pharmacokinetics; liver; hydrodynamics
HD-stimulated expression. The results of this structure–activity relationship study report new PEGylated polyacridine peptides that stabilize DNA polyplexes in the circulation for up to 5 h.

RESULTS
To advance the development of i.v. dosed nonviral gene delivery systems that mediated expression in the liver, we designed a series of PEGylated polyacridine peptides, each with four Acr residues and an increasing number of Lys residues (ranging from 1 to 6) separating Acr residues (Figure 1, structures 1–6). We hypothesized that increasing the spacing of Acr would increase binding affinity to DNA and potentially increase the in vivo stability and gene transfer efficacy of DNA polyplexes. The peptide series was designed based on results from our previous report that demonstrated maximal HD-stimulated gene transfer using PEGylated polyacridine peptide II, possessing 6 Acr spaced by single Lys residues (Figure 1, II). Based on these design criteria, and in effort to increase DNA-binding affinity while simultaneously decreasing the number of Acr residues, a polyacridine peptide series of (Acr-Lys\(_n\))\(_3\)-Acr-Lys-Cys-PEG was prepared where \(n = 1–6\) (Figure 1). By incorporating only four evenly spaced Acr residues the overall size of the polyacridine peptide series was decreased, and ranged from 9 to 24 residues. In addition to L-Lys polyacridine peptides, several D-Lys peptides were prepared (1D, 4D and 6D) using Fmoc-D-Lys(Boc) (Table 1). Likewise, an \(\varepsilon\)-acetyl Lys polyacridine control peptide (4Ac) was prepared using Fmoc-Lys(Ac) (Table 1). Peptides were purified by preparative reversed phase-high pressure liquid chromatography and characterized by liquid chromatography–mass spectrometry to establish their mass (Table 1). Each peptide possessed a C-terminal Cys residue that was modified with mPEG\(_{5kDa}\)-maleimide. Following PEGylation and reversed phase-high pressure liquid chromatography purification, PEGylated polyacridine peptides were characterized by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry to confirm this mass (Table 1). Based on our previous findings, the counter ions on PEGylated polyacridine peptides were converted from trifluoroacetic acid (TFA) to acetate to improve in vivo activity.\(^8\)

Properties of polyplexes and direct-HD dosing of PEGylated polyacridine peptide polyplexes
PEGylated peptides 1–6 are comparable in size (9–24 amino acids) to PEGylated peptide I (20 amino acids) and peptide II (13 amino acids) (Figure 1). PEGylated-Cys-Trp-Lys\(_{18}\) (I) lacks Acr and thereby binds to DNA exclusively through ionic interaction.\(^{18}\) Although peptide I has 18 Lys residues and forms polyplexes that remain stable during in vitro transfection,\(^{18,19}\) peptide I polyplexes rapidly dissociate in the circulation when dosed i.v., leading to the immediate metabolism of the plasmid by serum DNase.\(^{15,20}\)

![Figure 1](image-url) Structures of PEGylated polyacridine peptides. The structure of PEGylated polyacridine peptides 1–6 possessing four Lys-\(\varepsilon\)-acridines (Acr) and 1–6 spacing Lys residues are compared with PEGylated-Cys-Trp-Lys\(_{18}\) (I) and (Acr-Lys)\(_{6}\)-Cys-PEG (II). The structures of peptides ID, 1D, 4D and 6D possessing \(\varepsilon\)-Lys residues in place of L-Lys residues in peptides I, 1, 4 and 6 are not shown. The structure of control a peptide (4Ac) included N-acetyl groups capping each \(\varepsilon\)-amine of L-Lys residues in peptide 4.
are metabolically stable open polyplexes that at a lower stoichiometry of 0.2 nmol of peptide per g of DNA or less. Based on this result, and the binding equivalence determined by thiazole orange fluorescent dye displacement (Table 1). Peptide 1 and 1D both possessed equivalent low affinity for binding DNA whereas a modest affinity increase was revealed with the addition of each additional spacing Lys residues for peptides 2–6. Unexpectedly, Lys peptides 4 and 6 were found to bind pGL3 with lower affinity compared with 4 and 6. However, as anticipated, Lys-acetyl control peptide (4Ac) that possessed four Acr residues, each spaced by four acetylated Lys residues, established a rank priority in their apparent affinity for DNA to 1 h post DNA delivery.8

PEGylated peptides 1–6 were each able to bind to pGL3 and displace a thiazole orange fluorescent dye at an equivalence point ranging from 0.13 to 0.4 nmol of peptide per μg of pGL3, establishing a rank priority in their apparent affinity for DNA (Table 1). Peptide 1 and 1D both possessed equivalent low affinity for binding DNA whereas a modest affinity increase was revealed with the addition of each additional spacing Lys residues for peptides 2–6. Unexpectedly, Lys peptides 4 and 6 were found to bind pGL3 with lower affinity compared with 4 and 6. However, as anticipated, Lys-acetyl control peptide (4Ac) that possessed four Acr residues, each spaced by four acetylated Lys residues, possessed a lower apparent affinity.

We previously analyzed the quasi-elastic light scattering particle size and zeta potential of peptide II and 1 pGL3 polyplexes as a function of peptide to DNA stoichiometry to establish an asymptote at 0.8 nmols of peptide per μg of pGL3, establishing a rank priority for their apparent affinity for DNA (Table 1). Peptide 1 and 1D both possessed equivalent low affinity for binding DNA whereas a modest affinity increase was revealed with the addition of each additional spacing Lys residues for peptides 2–6. Unexpectedly, Lys peptides 4 and 6 were found to bind pGL3 with lower affinity compared with 4 and 6. However, as anticipated, Lys-acetyl control peptide (4Ac) that possessed four Acr residues, each spaced by four acetylated Lys residues, possessed a lower apparent affinity. We previously analyzed the quasi-elastic light scattering particle size and zeta potential of peptide II and 1 pGL3 polyplexes as a function of peptide to DNA stoichiometry to establish an asymptote at 0.8 nmols of peptide per μg of pGL3, establishing a rank priority for their apparent affinity for DNA (Table 1). Peptide 1 and 1D both possessed equivalent low affinity for binding DNA whereas a modest affinity increase was revealed with the addition of each additional spacing Lys residues for peptides 2–6. Unexpectedly, Lys peptides 4 and 6 were found to bind pGL3 with lower affinity compared with 4 and 6. However, as anticipated, Lys-acetyl control peptide (4Ac) that possessed four Acr residues, each spaced by four acetylated Lys residues, possessed a lower apparent affinity.

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![Figure 2](https://example.com/figure2.png)

**Figure 2.** Size and charge of PEGylated polyacridine peptide polyplexes. (a, b) Compare the mean particle size and zeta potential for PEGylated polyacridine peptide I, II, 1, 2, 3, 4, 5 and 6 polyplexes prepared at a fully saturated stoichiometry of 0.8 nmols of peptide per μg of pGL3 and a concentration of 30 μg of pGL3 per ml. The mean particle size and zeta potential of identically prepared control PEGylated polyacridine peptide ID, 1D, 4D, 4Ac and 6D polyplexes are compared in (c, d). The results establish the equivalent size and increasing charge for 1, 2, 3, 4, 5 and 6 polyplexes, and identify some differences between D versus L peptide polyplexes. The data are plotted as the mean and s.d. of multiple determinations.

| Polyacridine peptide and PEGylated polyacridine peptide structure | Synthetic yield (%) | Mass (calculated/observed) | Binding equivalent nmol μg⁻¹ of DNA |
|---------------------------------------------------------------|---------------------|-----------------------------|----------------------------------|
| (Acr-Lys)₃-Acr-Lys-Cys| 26                  | 1855.3/1855.6              | 0.4                              |
| 1. (Acr-Lys)₃-Acr-Lys-Cys-PEG | 55                  | 7355/7218                  | 0.4                              |
| (Acr-ollys)₃-Acr-ollys-Cys | 26                  | 1855.3/1855.8              | 0.4                              |
| 1D. (Acr-ollys)₃-Acr-ollys-Cys-PEG | 61                  | 7755/7500                  | 0.4                              |
| (Acr-Lys)₃-Acr-Lys-Cys | 10                  | 2239.9/2239.6              | 0.18                             |
| 2. (Acr-Lys)₃-Acr-Lys-Cys-PEG | 42                  | 7740/7684                  | 0.18                             |
| (Acr-Lys)₃-Acr-Lys-Cys | 19                  | 2624/2624.6                | 0.18                             |
| 3. (Acr-Lys)₃-Acr-Lys-Cys-PEG | 60                  | 8524/8316                  | 0.18                             |
| (Acr-Lys)₃-Acr-Lys-Cys | 20                  | 3008.9/3008.8              | 0.15                             |
| 4. (Acr-Lys)₃-Acr-Lys-Cys-PEG | 77                  | 8909/8697                  | 0.15                             |
| (Acr-ollys)₃-Acr-ollys-Cys | 26                  | 3008.9/3008.6              | 0.2                              |
| 4D. (Acr-ollys)₃-Acr-ollys-Cys-PEG | 56                  | 8909/8787                  | 0.2                              |
| (Acr-Aclys)₃-Acr-Aclys-Cys | 4                  | 3513.3/3512.8              | 0.15                             |
| 4Ac. (Acr-Aclys)₃-Acr-Aclys-Cys-PEG | 29                  | 9413/9131                  | 0.4                              |
| (Acr-Lys)₃-Acr-Lys-Cys | 19                  | 3393.4/3393.4              | 0.4                              |
| 5. (Acr-Lys)₃-Acr-Lys-Cys-PEG | 73                  | 9293/9202                  | 0.15                             |
| (Acr-Aclys)₃-Acr-Aclys-Cys | 18                  | 3777.9/3777.6              | 0.13                             |
| 6. (Acr-Lys)₃-Acr-Lys-Cys-PEG | 56                  | 9678/9332                  | 0.13                             |
| (Acr-ollys)₃-Acr-ollys-Cys | 77                  | 3777.9/3777.4              | 0.2                              |
| 6D. (Acr-ollys)₃-Acr-ollys-Cys-PEG | 47                  | 9678/9482                  | 0.2                              |

Abbreviation: PEG, polyethylene glycol. *Mass determined by positive mode electrospray ionization mass spectrometry. †Mass determined by positive mode matrix-assisted laser desorption/ionisation-time of flight mass spectrometry.
size (140–180 nm in diameter), but increase in charge incrementally from +5 to +21 mV, directly correlated with the Lys to PEG ratio (Figure 2b). Peptide 4Ac control polyplex possessed a similar particle size of 160 nm and a negative charge of −6 mV due to the lack of Lys ε-amine groups (Figures 2c and d). Compared with L-Lys peptides, D-Lys peptides 1D, 4D and 6D each produced larger polyplexes of 200–250 nm diameter (Figure 2c). Polyplexes prepared with 1D and 4D also possessed a much higher zeta potential (Figure 2d). The difference in polyplex physical properties resulted from weaker binding affinity, and contributed to lower gene expression for D-Lys peptide polyplexes as discussed below.

Direct-HD dosing (1 μg) of pGL3, polyplexes and lipoplex, followed by BLI measurement of luciferase in the liver at 24 h, was used to compare gene transfer efficiency (Figure 3). The level of expression mediated by peptide I or 4Ac polyplexes was indistinguishable from pGL3 (Figures 3a and b). Conversely, polyplexes prepared with peptides II and 1–6 increased gene transfer during direct-HD dosing (Figure 3a). A fivefold statistically significant increase in direct-HD dosing gene transfer efficiency over pGL3 was realized when using peptide 3 polyplexes (Figure 3a). These results suggest that short peptides that bind DNA exclusively by ionic binding (I) or polyintercalation (4Ac), easily dissociate from DNA resulting in a level of expression coincident to the direct-HD delivery of free pGL3.

In contrast to direct-HD dosing, HD-stimulated dosing with a short 5-min delay separating the primary dose and secondary stimulatory dose revealed that pGL3, peptide I, ID, PEI (P) and chitosan (C) polyplexes, in addition to Lipofectamine lipoplex (L) were all inactive in mediating gene transfer (Figures 3d–f). Not surprisingly, peptide I and ID polyplexes perform identically to naked pGL3 during direct HD and HD stimulation due to rapid dissociation of peptide I and ID in the blood, followed by rapid DNAse-mediated metabolism. Likewise, Lipofectamine lipoplexes also perform identically as naked DNA, producing a high level gene expression under direct HD and no expression under 5-min delayed HD stimulation, suggesting they also rapidly dissociate in the blood, leaving DNA susceptible to DNAse as has been reported previously.21

Unlike lipoplexes, PEI and chitosan polyplexes suppress direct-HD gene transfer efficiency by 10–100-fold relative to direct HD of pGL3, indicating greater polyplex stability (Figure 3a versus c). However, the complete loss of gene transfer activity by PEI and chitosan polyplexes under a short 5-min HD stimulation (Figure 3f) is consistent with their rapid removal from the circulation due to protein aggregation and filtration in the lung as has been reported.22

By comparison, polyplexes composed of PEGylated peptides 1–5 all produced a high level of gene expression under 5-min delay HD stimulation (Figure 3d). The high level of gene expression confirms that peptide 1–5 polyplexes are stable in the circulation. However, peptide 6 polyplexes were 15-fold less efficient at mediating gene transfer relative to peptide 5 polyplexes (Figure 3d). This could be due to either premature dissociation of the polyplex, protein binding followed by removal
from the circulation or incomplete intracellular DNA release. However, premature dissociation appears unlikely given the apparent high affinity of peptide 6 for pGL3 (Table 1).

Peptide 4D polyplexes resulted in a fivefold lower gene transfer efficiency compared with peptide 4 polyplexes, and peptide 6D polyplexes were 10-fold less efficient than peptide 6 polyplexes (Figures 3d and e). However, peptide 1 and 1D polyplexes were equivalent in gene transfer. Although there is apparently no enhancement in gene transfer realized by stabilizing polyacridine peptides to the action of proteases using D-Lys, the loss of gene transfer efficiency of 4D and 6D can be partially rationalized by considering their lower apparent binding affinity for DNA, resulting in larger particle size and higher zeta potential (Figures 2c and d), which would negatively influence biodistribution. We speculate that the lower DNA-binding affinity of 4 and 6D is due to the misalignment of Acr residues due to the reversed chirality of D-Lys residues.

Under HD-stimulated delivery, control peptide 4Ac polyplexes were ~100-fold less active in gene transfer relative to peptide 4 polyplexes (Figures 3d and e). This loss of activity is attributed to polyplex dissociation, as 4Ac binds DNA exclusively through polyintercalation with much less affinity than 4 (Table 1) and forms electronegative polyplexes (Figure 2d) that are less likely to experience altered biodistribution. This hypothesis is supported by the results of HD stimulation with extended time delay as discussed below.

A distinguishing feature of PEGylated polyacridine peptide polyplexes is their ability to mediate gene expression when dosed i.v. followed by an extended time delay before HD stimulation. In a previous study, PEGylated peptide II was used to form electronegative open polyplexes at 0.2 nmol µg⁻¹ of DNA and shown to mediate 10⁹ photons sec⁻¹ cm⁻² ser⁻¹ upon HD stimulation at a 1-h stimulation time, followed by a 100-fold decline at 2 h (Figure 4a).⁸ Anticipating that at DNA saturation (0.8 nmol to 1 µg pGL3), peptide II polyplexes may be even more protected from metabolism and mediate expression at prolonged stimulation times, we compared the level of luciferase expression at 0.2 and 0.8 nmol as a function of stimulation time. The results establish that 0.8 nmol of peptide remained full expression when applying HD stimulation at times up to 3 h, followed by a steady decline to near zero expression when applying HD stimulation at 7 h (Figure 4a).

Comparison of the HD-stimulated expression of pGL3 (1 µg) polyplexes mediated by 0.8 nmol of peptides 1–6 revealed a strong dependence on the number of Lys residues spacing Acr and the level of expression at extended delay times (Figure 4b). A single Lys in peptide 1 resulted in polyplexes that only produce 10⁸ photons sec⁻¹ cm⁻² ser⁻¹ at 1-h stimulation time, which decreases to zero by 3 h (Figure 4b). Two Lys residues in 2 improved the level of expression to 10⁹ at a 1-h HD-stimulation time, but still led to rapid loss of expression reaching zero by 3 h (Figure 4b). The addition of a third spacing Lys residue in 3 markedly improved the stability of HD-stimulated expression maintaining nearly 10⁹ photons sec⁻¹ cm⁻² ser⁻¹ at a 3-h HD-stimulation time, followed by a similar decrease to zero over 4 h. PEGylated polyacridine peptides 4 and 5 both mediated maximal HD-stimulated gene expression at delay times of 4–5 h, followed by a decrease in the level of HD-stimulated expression over 4 h. HD stimulation of pGL3 polyplexes prepared with 6 resulted in ~10-fold lower expression compared with 4 and 5, however, the level of HD-stimulated expression remained stable for 5 h before declining over 4 h (Figure 4b). The decreased level of HD-stimulated expression mediated with peptide 6 polyplexes under extended time delay is consistent with the results of HD stimulation at 5-min delay (Figure 3d).

There are several important conclusions that can be made from the analysis of the results presented in Figure 4. The addition of spacing Lys residues results in maintenance of maximal expression for longer HD-stimulation delay times. Although the addition of multiple Lys residues increases the peptide affinity for DNA through increased ionic binding, it is also clear that the combination of both polyintercalation and ionic binding are essential, and neither mode individually is sufficient. Polyplexes prepared with weak binding polyintercalating peptide 4Ac shows no expression at delay times of 1 h. Intermediate affinity peptides such as 1 or 2 produce a stimulation profile that rapidly declines to zero at 3-h delay times. Higher affinity peptides 3, 4 and 5 have stimulation profiles with sustained maximal expression for 3–5 h before declining steadily to zero over 4 h. The stimulation profile produced by peptide 6 polyplexes suggests its lower level of sustained expression is the result of incomplete intracellular release of DNA.

To investigate if the steady decline in HD-stimulated expression for peptide 4 polyplexes after 4 h was due to DNA metabolism in the blood, peptide 4 polyplexes were incubated for 24 h in heparinized whole mouse blood at 37°C, then dosed i.v., followed by HD stimulation after 1 h (Figure 5a). Comparison of polyplexes dosed immediately with those incubated in blood for 24 h established a 10-fold loss in HD-stimulated gene expression,
suggested that peptide 4 polyplexes were only partially digested by DNAses in the blood (Figure 5a).

A contributing factor to the steady decline of HD-stimulated expression over 4 h for peptides 3–6 polyplexes could also be the shedding of the PEGLylated peptide in the circulation, thereby exposing pGL3 to metabolism by DNAses. To test this hypothesis, a decay dose of plasmid expressing secreted alkaline phosphatase polyplex (9 μg) was used to form peptide 4 polyplexes that were co-administered with pGL3 (1 μg) peptide 4 polyplexes. HD stimulation at 9 h established a dose–response relationship in which maximal expression was restored at 9 μg of plasmid expressing secreted alkaline phosphatase polyplex (Figure 5b). Extending the HD-stimulation time delay from 7 to 24 h established that a decay dose of 9 μg of polyplex restored maximal expression at 7 and 9 h, which declined nearly 1000-fold by 12 h and diminished to zero at a HD stimulation delay time of 24 h (Figure 5c). These results support a hypothesis that shedding of PEGLylated polyacridine peptides during circulation results in exposure of DNA to DNAses. The decay dose should also shed peptide and could serve as a substrate to occupy DNAses in the circulation, delaying the metabolism of pGL3 polyplexes. HD stimulation of peptide II polyplexes prepared at 0.2 and 0.8 nmol of peptide per μg of pGL3 models this effect, with 0.2 nmol peptide II polyplexes resembling partially shed polyplexes that have HD-stimulation profiles shifted to the left relative to 0.8 nmol peptide II polyplexes (Figure 4a).

Pharmacokinetics and biodistribution of PEGLylated polyacridine peptide polyplexes

Following i.v. dosing, naked 125I-pGL3 is rapidly metabolized into fragments and eliminated from the blood (Figures 6a and c).8,15 The lack of DNA stability in blood account for the complete loss of HD-stimulated expression at 5 min post DNA delivery (Figure 3d). PEGLylated peptide 1 formed a more stable pGL3 polyplex with a longer T½-half-life and a delayed DNA metabolism in the circulation as demonstrated by gel electrophoresis and autoradiography of blood time points (Figures 6a and d). However, the weak binding affinity of peptide 1 results in low levels of HD-stimulated expression at 1 h post DNA delivery (Figure 4b).9 The improved stability of peptide 5 polyplexes in the circulation is evident by gel electrophoresis and autoradiography analysis of blood time points out to 2 h. Pharmacokinetic analysis of polyplexes prepared with peptides 2–6 established a correlation between the T½-half-life and mean residence time with increased protection of DNA from metabolism (Figure 6a, Table 2). The longest half-lives were determined for polyplexes prepared with peptides 4 and 6 (Figure 6a, Table 2), which are comparable to that achieved with DNA encapsulated in stealth liposomes.23 These results correlate with the HD-stimulation results in Figure 4b, indicating that greater DNA polyplex stability in the blood equates with maximum HD-stimulated expression at increasingly long stimulation times.

Biodistribution analysis established that each of the polyplexes produced a significant percent of dose associated with the liver at 5 min post DNA administration, ranging from 49 to 66% (Figure 6b, Table 3). Naked DNA is metabolized and eliminated from the liver such that only 6% of the 125I-pGL3 dose is recovered in the liver at 2 h (Figure 6b). Increasing the number of Lys residues in PEGLylated peptides 1–6 closely correlates with an increasing percentage of dose remaining in the liver at 2 h (Figure 6b). The liver and blood accounted for the majority of the 125I-pGL3 over time (Table 3). However, a slightly higher percentage of dose was recovered in lung for peptide 3 polyplexes, and in the spleen for peptide 1, 2, 3 and 4 and in the stomach for all polyplexes at 2 h, as a result of the liver metabolism.

DISCUSSION

The development of nonviral gene delivery systems that mediate expression in the liver following a conventional small volume i.v. dose of 1 μg of plasmid DNA polyplex is hampered by the lack of measurable expression for all delivery platforms studied to date. This has caused researchers to increase the DNA dose to 10–50 μg, allowing the detection of measurable, but low levels of luciferase in the liver.20,22,24–26 Generally, the expression levels have been difficult to compare due to the use of many different plasmids.
with different promoters and the lack of dose-matched comparison with direct-HD delivery. Furthermore, as has been noted previously, a 25-mg polyplex dose in mice equates to a 100-mg dose in humans, strongly suggesting that nonviral delivery systems possessing greatly improved efficiency need to be developed.

We have developed a standardized protocol to compare the level of expression mediated by i.v. dosed pGL3 polyplexes with dose-matched direct-HD delivery of pGL3. The use of a calibrated BLI measurement for quantifying luciferase in the liver of albino ICR mice following a 1-mg dose of pGL3 or pGL3 polyplex, allows for a direct efficiency comparison.

The protocol described aims to optimize in vivo delivery of DNA polyplexes. Although PEGylated polyacridine polyplexes can be stimulated to express in vivo, they do not transfect cells in culture under standard in vitro gene transfer protocols. This is primarily the result of stealthing of the polyplex by PEG, which blocks it from binding to cells, whereas substitution of a polyacridine peptide with the fusogenic peptide melittin results in polyplexes with potent in vitro gene transfer properties. Following the i.v. administration of PEGylated polyacridine pGL3 polyplexes there is also no detectable expression without application of HD stimulation. However, with the application of HD stimulation at time delays of 5 min or longer, certain pGL3 polyplexes express luciferase as efficiently as direct-HD administration of pGL3. The results suggest that HD-stimulation transports pGL3 polyplexes in the blood or liver into the nuclei of hepatocytes leading to the observed gene expression. The magnitude of expression following

Figure 6. Pharmacokinetics and biodistribution of PEGylated polyacridine peptide polyplexes. (a) Illustrates the pharmacokinetic profile for 125I-pGL3 and 125I-pGL3 polyplexes prepared with peptides 1, 2, 3, 4, 5 and 6. The rapid loss of 125I-pGL3 and 125I-pGL3 peptide 1 polyplexes is due to metabolism as reported previously. The pharmacokinetic profile for peptide 2–6 polyplexes is consistent with their DNAse stability in the circulation. The pharmacokinetic time points from (a) were analyzed by agarose gel electrophoresis and autoradiography for DNA (c), peptide 1 polyplexes (d) and peptide 5 polyplexes (e). (b) Illustrates the biodistribution of 125I-pGL3 and 125I-pGL3 polyplexes in the liver at times ranging from 5 min to 6 h. The results represent the mean and s.d. of triplicate mice. The derived pharmacokinetic parameters are presented in Table 2, and the complete tissue biodistribution results are included in Table 3.

| Table 2. PEGylated polyacridine peptide polyplex pharmacokinetics |  |
|---|---|---|---|---|---|---|---|---|---|
| PEGylated polyacridine peptide polyplex | t_{1/2}^a (min) | t_{1/2}^b (min) | V_d (ml) | Cl (ml min⁻¹) | MRT (min) | AUC (µg min ml⁻¹) |
| 1. (Acr-Lys)₃-Acr-Lys-Cys-PEG/125I-DNA | 3.4 ± 0.6 | 112.1 ± 28.3 | 357.1 ± 41.3 | 2.3 ± 0.4 | 162.5 ± 34.6 | 67.2 ± 10.3 |
| 2. (Acr-(Lys 2)₃-Acr-Lys-Cys-PEG/125I-DNA | 2.1 ± 0.7 | 231.6 ± 53.9 | 289.5 ± 3.8 | 0.9 ± 0.2 | 326.3 ± 79.1 | 172.3 ± 38.1 |
| 3. (Acr-(Lys 3)₃-Acr-Lys-Cys-PEG/125I-DNA | 2.1 ± 0.4 | 375.4 ± 91.2 | 221.1 ± 17.3 | 0.4 ± 0.1 | 545.8 ± 128.4 | 365.3 ± 65.4 |
| 4. (Acr-(Lys 4)₃-Acr-Lys-Cys-PEG/125I-DNA | 4.1 ± 2.2 | 373.6 ± 44.3 | 159.1 ± 9.4 | 0.3 ± 0.0 | 540.3 ± 62.3 | 507.7 ± 43.2 |
| 5. (Acr-(Lys 5)₃-Acr-Lys-Cys-PEG/125I-DNA | 3.2 ± 1.7 | 260.5 ± 87.7 | 333.6 ± 16.8 | 0.9 ± 0.4 | 371.6 ± 133.7 | 169.3 ± 57.1 |
| 6. (Acr-(Lys 6)₃-Acr-Lys-Cys-PEG/125I-DNA | 3.8 ± 1.6 | 291.1 ± 97.4 | 178.3 ± 32.3 | 0.5 ± 0.1 | 420.6 ± 139.6 | 352.8 ± 92.1 |

Abbreviations: MRT, mean residence time; PEG, polyethylene glycol. aCalculated using blood cpm values over 120 min, assuming complete DNA stability. bCalculated α-half-life. cCalculated β-half-life. dVolume of distribution. eTotal body clearance rate. fMean residence time. gArea under the curve.
biodistribution that decreases the amount of DNA polyplex relative to 4 and 6 (Table 1), a larger particle size for 1D, 4D and 6D relative to 1, 4 and 6 (Figures 2a and c) and a higher zeta potential for 1D and 4D relative to 1 and 4 (Figures 2b and d). Therefore, the differences in DNA binding between I and D peptides, leading to both larger size and higher charge, likely result in changes in biodistribution that decreases the amount of DNA polyplex available for HD-stimulated expression.

Extending the time delay by which HD-stimulated expression leads to maximal expression is important toward achieving targeted delivery of polyplexes and expression in tissues outside the liver. The shedding of PEGylated peptides from pGL3 polyplexes results in rapid deactivation of the DNA due to the action of DNase in the blood and liver. This is evident with PEGylated Cys-Trp-Lys18 (I), which rapidly dissociates in the circulation. The ability to dramatically extend maximal HD-stimulated expression to 9 h by administering a decy dose of plasmid expressing secreted alkaline phosphatase polyplex (Figures 5b and c) provides another important clue as how to improve nonviral gene delivery systems. This may be the result of saturating DNAse in the blood29 or by blocking uptake of the scavenger receptor as has been reported previously.29 Interestingly, the DNase protection afforded to pGL3 by PEGylated polyacridine peptides in blood is not as critical as when dosing polyplexes via local administration, such as intramuscular electroporation.28

Polyplexes or lipoplexes prepared with PEI, Lipofectamine, chitosan or PEGylated polysine peptide I, all fail to mediate HD-stimulated expression even at 5 min (Figure 3d and f). These results are consistent with prior studies that examined the stability of lipoplexes in the blood and the pharmacokinetics and biodistribution of PEI and chitosan polyplexes.2,23,30,35

In conclusion, we have not only significantly improved the design of PEGylated polyacridine peptides by increasing the spacing between Acr residues resulting in more efficient gene delivery, but have also demonstrated a process by which nonviral
gene delivery systems can be systematically optimized in vivo. In addition to Acr spacing, other subtle differences in the linkage between PEG and a polycridine peptide have an important role in HD-stimulated expression. The detailed report of these studies will be the subject of a future correspondence.

MATERIALS AND METHODS

Unsubstituted Wang resin, 9-hydroxybenzotriazole, Fmoc-protected amino acids, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluoro-

phosphate (HATU), 5-[3-(2-carboxyethyl)-phosphino]hydrochloride, 9-chloroacridine and thiazole orange were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Agarose was obtained from Gibco-BRL (Carlsbad, CA, USA), mPEG-maleimide (5000 Da) was purchased from Laysan Bio (Arab, AL, USA). 9-Phenoxyacridine and Fmoc-Lysine(Acr)-OH were prepared as recently reported.25,26 The polycridine peptides defined in Figure 1 and Table 1 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. The reaction yields were improved by activating amino acids with 9-hydroxybenzotriazole and HATU while using double coupling of Fmoc-Lys(Acr)-OH and triple coupling for the spacing amino acid, using a fivelfold excess of amino acid over resin. Peptides were removed from resin and side chain deprotected using a cleavage cocktail of TFA/ethanedithiol/water (93:4:3 v/v/v) for 3 h followed by precipitation in cold ether. Precipitates were centrifuged for 10 min at 5000 × g at 4 °C and the supernatant decanted. Peptides were then reconstituted with 0.1 v/v % TFA and purified to homogeneity on reversed phase-high pressure liquid chromatography by injecting 0.5–2 μmol onto a Vydac C18 (Grace Davison Discovery Sciences, Deerfield, IL, USA) semi-preparative column (2 × 25 cm) eluted at 10 ml min⁻¹ with 0.1 v/v % TFA with an acetonitrile gradient of 15–25 v/v % over 30 min while monitoring acridine at 409 nm. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized and stored at −20 °C. Purified peptides were reconstituted in 0.1 v/v % TFA and quantified by absorbance (acridine ε₂̂̂̂̂₄₀₉nm = 9266 M⁻¹·cm⁻¹) assuming additivity of ε for multiple acridines) to determine isolated yield (Table 1). Purified peptides were characterized by liquid chromatography–mass spectrometry by injecting 2 nmol onto a Vydac C18 analytical column (0.47 × 25 cm) eluted at 1 ml min⁻¹ with 0.1 v/v % TFA and an acetonitrile gradient of 15–45 v/v % over 30 min while acquiring electrospray ionization mass spectrometry in the positive mode.

Synthesis and characterization of PEGylated polycridine peptides

PEGylation of the Cys residue on (Acr-Lys)n-Acr-Lys-Cys (where n = 1–6) was achieved by reacting 1 μmol of peptide with 1.1–1.5 μmol of PEG5kDa-maleimide in 4 ml of 100 mM 4-(2-hydroxyethyl)-1-piperazinethane-sulfonic acid (HEPES) buffer pH 7.5 for 12 h at room temperature. PEGylated peptides were purified by semi-preparative reversed phase-high pressure liquid chromatography eluted with 0.1 v/v % TFA with an acetonitrile gradient of 20–60 v/v % acetonitrile while monitoring acridine at 409 nm. The major peak was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized and stored at 20 °C. The counter ion was exchanged by chromatography on a G-25 column (2.5 × 50 cm) equilibrated with 0.1 v/v % acetic acid to obtain the peptide in an acetic salt form. The major peak corresponding to the PEG peptide eluted in the void volume (100 ml) was pooled, concentrated by rotary evaporation, freeze dried and the PEG content determined by using an APEX 396 synthesizer (Advanced ChemTech, Louisville, KY, USA) operated in the positive ion mode. The target and ionized on a Bruker Biflex III Mass Spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) operated in the positive ion mode.

Formulation and characterization of PEGylated polycridine peptide polyplexes

The relative binding affinities of PEGylated polycridine peptides for pGL3 was determined by a fluorescent coassay.27 PGL3 (200 μl of 5 μg ml⁻¹ in 5 μl HEPES pH 7.5 containing 0.1 μl thiazole orange) was mixed with 0.0, 0.05, 0.1, 0.13, 0.25, 0.2, 0.25, 0.3, 0.4, 0.45 or 1 mmol of PEGylated polycridine peptide in 300 μl of HEPES and allowed to bind at room temperature for 30 min. Thiazole orange fluorescence was measured using an LS50B fluorometer (Perkin-Elmer, Cambridge, UK) by exciting at 498 nm while monitoring emission at 546 nm with the slit widths set at 10 nm. A fluorescence blank of thiazole orange in the absence of pGL3 was subtracted from all values before data analysis. The binding equivalent was defined as the peptide stoichiometry that maximally decreased thiazole orange fluorescence.

The particle size and zeta potential were determined by preparing 2 ml of polyplex in 5 ml HEPES pH 7.5 at a pGL3 concentration of 30 μg ml⁻¹ and a PEGylated polycridine peptide stoichiometry of 0.8 mmol μg⁻¹ of pGL3. The particle size was measured by quasi-elastic light scattering at a scatter angle of 90° on a Brookhaven ZetaPlus particle sizer (Brookhaven institute Corporation, Holtsville, NY, USA). The zeta potential was determined as the mean of 10 measurements immediately following acquisition of the particle size.

Pharmacokinetic analysis of PEGylated polycridine peptide polyplexes

Radiiodinated pGL3 was prepared as previously described.38 Triplicate mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (100 mg kg⁻¹) and xylazine hydrochloride (10 mg kg⁻¹), then underwent a dual cannulation of the right and left jugular veins. An i.v. dose of 1215I-pGL3 (3 μg, 1.2 μCi in 50 μl of HEPES-buffered mannitol (5 μM HEPES, 0.27 mM mannitol and pH 7.4) or 125I-pGL3 polyplex (3 μg) was administered via the left catheter, and blood samples (10 μl) were drawn from the right catheter at 1, 3, 6, 10, 20, 30, 60, 90 and 120 min and immediately frozen, then replaced with 10 μl of normal saline. The amount of radioactivity in each blood time point was quantified by direct γ-counting. Blood time points were digested with proteinase K for 12 h and polyplexes were extracted with 500 μl of phenol/chloroform/soybean alcohol (24:25:1) to remove PEGylated peptides, followed by precipitation of DNA with the addition of 1 ml of ethanol.6,15 The precipitate was collected by centrifugation at 13 000 × g for 10 min, and the DNA pellet was dried and dissolved in 5 mM HEPES buffer pH 7.4. DNA samples were combined with 2 μl of loading buffer and applied to a 1% agarose gel (50 ml) and electrophoresed in Tris-borate-EDTA buffer at 70 V for 60 min.15 The gel was dried on a zeta probe membrane and autoradiographed on a Phosphor Imager (Molecular Devices, Sunnyvale, CA, USA) following a 15 h exposure.

Biodistribution analysis of PEGylated polycridine peptide polyplexes

125I-pGL3 (1.5 μg in 50 μl of HEPES-buffered mannitol, 0.6 μCi) or 125I-pGL3 polyplexes (1.5 μg) were diluted in triplicate mice in the tail vein. At times ranging from 5 min to 6 h, mice were anesthetized by intraperitoneal injection of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹) and then killed by cervical dislocation. The major organs (liver, lung, spleen, stomach, kidney, heart, large intestine and small intestine) were harvested, rinsed with saline, and the radioactivity in each organ was determined by direct γ-counting and expressed as the percentage of the dose in the organ.

Direct HD dosing and HD-stimulated expression

pGL3 (1 μg), pGL3 PEGylated polycridine polyplexes (0.8 mmol μg⁻¹ of PGL3), PEI PEI (NP of 5) or chitosan (NP of 20) polyplexes, or pGL3 Lipofectamine (2:1 Lipid:pGL3 wt ratio) lipoplexes were prepared in a volume of normal saline corresponding to 9 wt/vol% of the mice’s body weight (1.8–2.25 ml based on 20–25 g mice). pGL3, polyplexes or lipoplexes were directly HD dosed by administering the 1.8–2.25 ml volume to the tail vein of triplicate mice in 5 s according to a published procedure.12,39 HD-stimulated expression was performed by tail vein dosing triplicate mice with 1 μg of pGL3, PEGylated polycridine polyplexes, PEI polyplex, chitosan polyplex or Lipofectamine lipoplex in 50 μl of HEPES-buffered mannitol. At times ranging from 5 min to 9 h, a HD-stimulatory dose of...
normal saline (9 wt/vol% of the body weight) was administered over 5 s. At 24 h post HD stimulation, mice were anesthetized by 3% isoflurane, then administered an intraperitoneal dose of 80 μl (2.4 mg) of d-luciferin (30 μg · μl⁻¹ in phosphate-buffered saline). At 5 min following the d-luciferin dose, mice were imaged for BLI on an IVIS Imaging 200 Series (Xenogen, Hopkins, MA, USA). BLI was performed in a light-tight chamber on a temperature-controlled, adjustable stage while isoflurane was administered by a gas manifold at a flow rate of 3%. Images were acquired at a ‘medium’ binning level and a 24.6-cm field of view with 10 s acquisition time. The Xenogen system reported bioluminescence as photons sec⁻¹ cm⁻² ster₃ in a 2.86-cm diameter region of interest covering the liver. The integration area was transformed to pmols.l

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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