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High-Affinity PEGylated Polyacridine Peptide Polyplexes Mediate Potent In Vivo Gene Expression

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Abstract

PEGylated 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 (Acr-Lys)$_6$-Cys-PEG$_{5kDa}$ stabilizes a 1 μg pGL3 dose for up to 1 hr 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 (Fernandez C.A. et al. Gene Therapy 2011). In the present study we report that increasing the spacing of Acr with either 4 or 5 Lys residues, dramatically increases the stability of PEGylated polyacridine peptide polyplexes in the circulation allowing maximal HD-stimulated expression for up to 5 hrs 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 hrs. This structure-activity relationship study defines the PEGylated polyacridine peptide requirements for maintaining fully transfection competent plasmid DNA in the circulation for 5 hrs and provides an understanding as to why polyplexes or lipoplexes prepared with PEI, chitosan or Lipofectamine are inactive within 5 min following i.v. dosing.

Keywords

gene delivery; peptide; pharmacokinetics; liver; hydrodynamics

Introduction

The development of highly efficient nonviral gene delivery systems that mediate expression in animals following i.v. dosing remains a significant challenge[1]. Delivery systems that are optimized *in vitro* often fail to mediate expression *in vivo* due to a combination of aberrant pharmacokinetics or biodistribution[2], inadequate intracellular trafficking or DNA release, [3-5] or the inability to penetrate the nucleus[6,7].

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

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tail vein of mice. Following a delay time of 5 min to 9 hrs, 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 liver is measured by quantitative bioluminescence imaging (BLI) at 24 hrs. 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 [8,9].

PEI or Lipofectamine mediated gene transfer have emerged as benchmark delivery agents for comparison with developmental *in vitro* gene transfer agents[10,11]. Similarly, direct hydrodynamic (HD) dosing is the most efficient benchmark delivery method to calibrate the development of *in vivo* gene transfer agents[12]. Direct-HD delivery of 10 ng to 5 μg of pGL3 in mice produces a linear BLI calibration curve covering a dynamic range of 5-orders of magnitude(10^5-10^{10} photons/sec/cm^2/ser)[13,14]. While *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 prior to an HD-stimulatory dose of saline, results in no detectable luciferase expression, due to the rapid metabolism of DNA in the blood [8,15].

PEGylated glycoproteins were the first gene delivery systems that produced measurable luciferase expression following HD-stimulation[9]. However, the level of expression was very low (10^6 photons/sec/cm^2/ser) 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[8]. Modification of the ε-amine of Fmoc-Lys with acridine affords an amino acid (Acr) that is incorporated into a peptide during solid phase synthesis[16,17]. We reported that increasing the number of Acr residues from 2-6 greatly increases the binding affinity of polyacridine peptides for DNA[8]. 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 hr post-DNA delivery, $(Acr\text{-}Lys)_6$-Cys-PEG_{5KDa} pGL3 (1 μg) polyplexes mediated luciferase expression equivalent in magnitude to direct-HD dosing of 1 μg of pGL3[8].

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 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 hrs.

Results

To advance the development of i.v. dosed nonviral gene delivery systems that mediated expression in liver, we designed a series of PEGylated polyacridine peptides, each with four Acr residues and an increasing number of Lys residues (ranging from 1-6) separating Acr residues (Fig. 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 (Fig. 1, **II**)[8]. 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** (Fig. 1). By incorporating only four evenly spaced Acr residues the overall size of the polyacridine peptide series was decreased, and ranged from 9-21 residues. In addition to L-Lys polyacridine peptides, several D-Lys peptides were prepared (**1D**, **4D**, **6D**) using Fmoc-D-Lys(Boc) (Table 1). Likewise, an ε-acetyl Lys polyacridine control peptide (**4Ac**) was prepared using FMOC-Lys(Ac) (Table 1). Peptides were purified by preparative RP-HPLC and characterized by LC-MS to establish their mass (Table 1). Each peptide possessed a C-terminal Cys residue which was modified with $mPEG_{5kDa}$-maleimide. Following PEGylation and RPHPLC purification, PEGylated polyacridine peptides were characterized by MALDI-TOF to confirm this mass (Table 1). Based on our previous findings, the counter ions on PEGylated polyacridine peptides were converted from 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**)** (Fig. 1). PEGylated-Cys-Trp-Lys_{18} (**I**) lacks Acr and thereby binds to DNA exclusively through ionic interaction[18]. While peptide **I** has eighteen 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[9,15,20]. In contrast, peptide **II**, possessing six Acr and only six Lys residues, binds DNA with a much higher affinity than **I** due to its combined polyintercalation and ionic interaction[8]. Consequently, peptide **II** polyplexes are stable in the circulation for up to 2 hrs, and thereby mediate maximal luciferase expression upon HD-stimulation for up to 1 hr post-DNA delivery[8].

PEGylated peptides **1-6** were each able to bind to pGL3 and displace a thiazole fluorescent dye at an equivalence point ranging from 0.13-0.4 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, D-Lys peptides **4D** and **6D** were found to bind pGL3 with lower affinity compared to **4** and **6**. However, as anticipated, Lys-acetyl control peptide (**4Ac**) which

possessed four Acr residues, each spaced by four acetylated Lys residues, possessed a lower apparent affinity.

We previously analyzed the quasi-elastic light scattering (QELS) 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 DNA or less[8]. Based on this result, and the binding equivalence determined by thiazole fluorescent dye displacement (Table 1), the peptide to DNA stoichiometry was kept constant at 0.8 nmol of peptide per μg of pGL3 to allow direct comparison of the physical and biological properties of each peptide polyplex. The size and charge of pGL3 polyplexes prepared with peptides **I**, **II,** and **1**-**6** established a mean diameter that ranged from 140-180 nm (Fig. 2A) and a zeta potential ranging from −3 to +21 mV (Fig. 2B).

Peptides **I** and **II** both produced electro-positive polyplexes of approximately 150 nm diameter. Since polyplex surface charge depends on the Lys to PEG ratio, peptide **I** polyplexes possessed a higher zeta potential of +18 mV compared to peptide **II** polyplexes with zeta potential of +8 mV (Fig. 2B). We have previously shown that at a lower stoichiometry of 0.2 nmol of peptide per μg of pGL3, peptide **II** forms metabolically stable open-polyplexes that are −5 mV in charge[8]. Similarly, even at full saturation of pGL3, peptide **1** forms 160 nm polyplexes with a charge of −3 mV (Fig 2B)[8]. By comparison, peptides **2**-**6** polyplexes are of similar size (140-180 nm in diameter), but increase in charge incrementally from +5 to +20 mV, directly correlated with the Lys to PEG ratio (Fig. 2B). The 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 (Fig. 2C and D). Compared to L-Lys peptides, D-Lys peptides **1D**, **4D** and **6D** each produced larger polyplexes of 200-250 nm diameter (Fig. 2C). Polyplexes prepared with **1D** and **4D** also possessed a much higher zeta potential (Fig. 2D). The difference in polyplex physical properties resulted from weaker binding affinity, and contributed to lower gene expression for DLys peptide polyplexes as discussed below.

Direct-HD dosing (1 μg) of pGL3, polyplexes and lipoplex, followed by BLI measurement of luciferase in liver at 24 hrs, was used to compare gene transfer efficiency (Fig. 3). The level of expression mediated by peptide **I** or **4Ac** polyplexes was indistinguishable from pGL3 (Fig. 3A and B). Conversely, polyplexes prepared with peptides **II** and **1**-**6** increased gene transfer during direct-HD dosing (Fig. 3A). A 5-fold statistically-significant increase in direct-HD dosing gene transfer efficiency over pGL3 was realized when using peptide **3** polyplexes (Fig. 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 (**L**) lipoplexes were all inactive in mediating gene transfer (Fig. 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 (Fig. 3A versus C). However, the complete loss of gene transfer activity by PEI and chitosan polyplexes under a short 5 min HD-stimulation (Fig. 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 (Fig. 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 (Fig. 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 5-fold lower gene transfer efficiency compared to peptide **4** polyplexes, and peptide **6D** polyplexes were 10-fold less efficient than peptide **6** polyplexes (Fig. 3D and E). However, peptide **1** and **1D** polyplexes were equivalent in gene transfer. While 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 (Fig. 2C and D), which could negatively influence biodistribution. We speculate that the lower DNA binding affinity of **4D** 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 approximately 100-fold less active in gene transfer relative to peptide **4** polyplexes (Fig. 3D and E). This loss of activity is attributed to polyplex dissociation, since **4Ac** binds DNA exclusively through polyintercalation with much less affinity than **4** (Table 1) and forms electronegative polyplexes (Fig. 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 prior to HD-stimulation. In a previous study, PEGylated peptide **II** was used to form electronegative open-polyplexes at 0.2 nmol per μg of DNA and shown to mediate 10^8 photon/sec/cm^2/ser upon HD-stimulation at a 1 hr stimulation time, followed by a 100-fold decline at 2 hrs (Fig 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 nmols as a function of stimulation time. The results establish that 0.8 nmol of peptide maintained full expression when applying HD-stimulation at times up to 3 hrs, followed by a steady decline to near zero expression when applying HD-stimulation at 7 hrs (Fig. 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 (Fig. 4B). A single Lys in peptide **1** resulted in polyplexes that only produce 10^7 photons/sec/cm^2/ser at 1 hr stimulation time, which decreases to zero by 3 hrs (Fig. 4B). Two Lys residues in **2** improved the level of expression to 10^8 at a 1 hr HD-stimulation time, but still led to rapid loss of expression reaching zero at by 3 hrs (Fig. 4B). The addition of a third spacing Lys residue in **3** dramatically improved the stability of HD-stimulated expression maintaining nearly 10^8 photons/sec/cm^2/ser at a 3 hr HD-stimulation time, followed by a similar decrease to zero over 4 hrs. PEGylated polyacridine peptides **4** and **5** both mediated maximal HD-stimulated gene expression at delay times of 4-5 hrs, followed by a decrease in the level of HD-stimulated expression over 4 hrs. HD-stimulation of pGL3 polyplexes prepared with **6** resulted in approximately 10-fold lower expression compared to **4** and **5**, however the level of HD-stimulated expression remained stable for 5 hrs before declining over 4 hrs (Fig. 4B). The decreased level of HD-stimulated gene expression mediated with peptide **6** polyplexes under extended time delay is consistent with the results of HD-stimulation at 5 min delay (Fig. 3D).

There are several important conclusions that can be made from 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. While 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 hr. Intermediate affinity peptides such as **1** or **2** produce a stimulation profile that rapidly declines to zero at 3 hr delay times. Higher affinity peptides **3**, **4** and **5** have stimulation profiles with sustained maximal expression for 3-5 hrs before declining steadily to zero over 4 hrs. 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 hrs was due to DNA metabolism in the blood, peptide **4** polyplexes were incubated for 24 hrs in heparinized whole mouse blood at 37°C, then dosed i.v., followed by HD-stimulation after 1 hr (Fig. 5A). Comparison of polyplexes dosed immediately with those incubated in blood for 24 hrs established a 10-fold loss in HD-stimulated gene expression, suggesting that peptide **4** polyplexes were only partially digested by DNAse in the blood (Fig. 5A).

A contributing factor to the steady decline of HD-stimulated expression over 4 hrs for peptides **3**-**6** polyplexes could also be the shedding of the PEGylated peptide in the circulation, thereby exposing pGL3 to metabolism by DNAse. To test this hypothesis, a decoy dose of pSEAP (1.5, 4 and 9 μg) was used to form peptide **4** polyplexes that were co-administered with pGL3 (1 μg) peptide **4** polyplexes. HD-stimulation at 9 hrs established a dose-response relationship in which maximal expression was restored at 9 μg of pSEAP polyplex (Fig. 5B). Extending the HD-stimulation time delay from 7 to 24 hrs established that a decoy dose of 9 μg of polyplex restored maximal expression at 7 and 9 hrs, which declined nearly 1000-fold by 12 hrs and diminished to zero at a HD stimulation delay time of 24 hrs (Fig. 5C). These results support a hypothesis that shedding of PEGylated polyacridine peptides during circulation results in exposure of DNA to DNAse. The decoy dose should also shed peptide and could serve as a substrate to occupy DNAse 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 (Fig. 4A).

Pharmacokinetics and Biodistribution of PEGylated Polyacridine Peptide Polyplexes

Following i.v. dosing, naked ^{125}I-pGL3 is rapidly metabolized into fragments and eliminated from the blood (Fig. 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 (Fig. 3D). PEGylated peptide **1** formed a more stable pGL3 polyplex with a longer α-half-life and a delayed DNA metabolism in the circulation as demonstrated by gel electrophoresis and autoradiography of blood time points (Fig. 6A and D). However, the weak binding affinity of peptide **1** results in low levels of HD-stimulated expression at 1 hr post-DNA delivery (Fig. 4B)[8]. 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 hrs. Pharmacokinetic analysis of polyplexes prepared with peptides **2**-**6** established a correlation of between the β-half-life and MRT with increased protection of DNA from metabolism (Fig. 6A, Table 2). The longest half-lives were determined for polyplexes prepared with peptides **4** and **6** (Fig. 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 49-66% (Fig. 6B, Table 3). Naked DNA is metabolized and eliminated from the liver such that only 6% of the ^{125}I-pGL3 dose is recovered in liver at 2 hrs (Fig. 6B). Increasing the number of Lys residues in PEGylated peptides **1**-**6** closely correlates with an increasing percentage of dose remaining in liver at 2 hrs (Fig. 6B). The liver and blood accounted for the majority of the ^{125}I-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 hrs, as a result of 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[2], a 25 μg 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 μg dose of pGL3 or pGL3 polyplex, allows for a direct efficiency comparison.

The protocol described aims to optimize *in vivo* delivery of DNA polyplexes. While 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 [18], whereas substitution of a polyacridine peptide with the fusogenic peptide melittin results in polyplexes with potent *in vitro* gene transfer properties[27]. 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 a variable delay-time between pGL3 polyplex dose and HD-stimulation is a parameter used to estimate polyplex stability. Polyplexes with minimal stability mediate relatively low levels of expression at very short HD-stimulated delay-times of 5 min due to metabolism of DNA in the blood[9]. Conversely, polyplexes that are optimally stabilized result in maximal expression for prolonged HD-stimulated delay times of 4 to 5 hrs. Polyplexes that exceed optimal stability fail to release DNA and produce lower levels of expression for prolonged HD-stimulation delay times. This delicate balance between polyplex stability and release has been noted as key barrier to achieving efficient nonviral delivery [5,22].

PEGylated polyacridine peptide polyplexes are unique in mediating efficient HD-stimulated gene expression at extended delay times due to the ability to adjust their affinity for DNA by changing the number of Acr and Lys residues. A significant finding of this study is that spacing Acr with additional Lys residues significantly extends the delay times. PEGylated polyacridine peptides with 4 Acr and either 4 or 5 spacing Lys residues (peptides **4** and **5)** form polyplexes that circulate and maintain HD-stimulated expression for up to 4-5 hrs (Fig. 4B). By comparison, peptide **II** possessing 6 Acr spaced by a single Lys residue resulted in a

maximal expression for up to 3 hrs in the circulation (Fig. 4A). It is also clear, by comparison of HD-stimulated expression using polyplexes prepared with peptide **I**, **4Ac** and **4**, that a combination of intercalator and ionic binding to DNA is necessary to achieve sufficient polyplex stability to mediate HD-stimulated expression.

An unexpected finding was the difference between L-Lys and D-Lys peptide polyplexes. Peptides **4D** and **6D** polyplexes resulted in a 10-fold loss in expression relative to peptides **4** and **6** polyplexes when delivered by direct-HD (Fig. 3A and B) or HD-stimulation (Fig. 3D and E). This result correlates with the apparent lower affinity of peptides **4D** and **6D** for binding DNA relative to **4** and **6** (Table 1), a larger particle size for **1D**, **4D** and **6D** relative to **1**, **4**, and **6** (Fig. 2A and C), and a higher zeta potential for **1D** and **4D** relative to **1** and **4** (Fig. 2B and D). Therefore, the differences in DNA binding between L 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-Lys_{18} (**I**), which rapidly dissociates in the circulation. The ability to dramatically extend maximal HD-stimulated expression to 9 hrs by administering a decoy dose of pSEAP polyplex (Fig. 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 blood[2] or by blocking uptake of the scavenger receptor as has been reported previously [28]. 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 intramuscle electroporation [29].

Polyplexes or lipoplexes prepared with PEI, Lipofectamine, chitosan, or PEGylated polylysine peptide **I,** all fail to mediate HD-stimulated expression even at 5 min (Fig. 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 polyacridine peptide play 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-Azabenzo-triazol-1-yl)-N,N,N’,N’-tetramethylurionium hexafluorophosphate (HATU),

Fmoc-Lysine-OH, and N-Methyl-2-pyrrolidone (NMP) were obtained from AAPPTec (Louisville, KY). N,N-Dimethylformamide (DMF), trifluoroacetic acid (TFA), and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Diisopropylethylamine, piperidine, acetic anhydride, Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 9-chloroacridine and thiazole orange were obtained from Sigma Chemical Co. (St. Louis, MO). Agarose was obtained from Gibco-BRL. mPEG-maleimide (5,000 Da) was purchased from Laysan Bio (Arab, AL). DLuciferin and luciferase from *Photinus pyralis* were obtained from Roche Applied Science (Indianapolis, IN). pGL3 control vector, a 5.3 kbp luciferase plasmid containing a SV40 promoter and enhancer, was obtained from Promega (Madison, WI). pGL3 was amplified in a DH5α strain of *Escherichia coli* and purified according to the manufacturer's instructions.

Synthesis and Characterization of Polyacridine Peptides

9-Phenoxyacridine and Fmoc-Lysine(Acridine)-OH were prepared as recently reported[27,36]. The polyacridine 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 with standard Fmoc procedures. The reaction yields were improved by activating amino acids with 9-hydroxybenzotriazole and HATU while employing double coupling of Fmoc-Lys(Acr)-OH and triple coupling for the spacing amino acid, using a 5-fold 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 hrs followed by precipitation in cold ether. Precipitates were centrifuged for 10 min at 5000 x g at 4°C and the supernatant decanted. Peptides were then reconstituted with 0.1 v/v % TFA and purified to homogeneity on RP-HPLC by injecting 0.5-2 μmol onto a Vydac C18 semi-preparative column (2 × 25 cm) eluted at 10 ml per min 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 $\varepsilon_{409\ nm} = 9266\ M^{-1}\ cm^{-1}$ assuming additivity of ε for multiple acridines) to determine isolated yield (Table 1). Purified peptides were characterized by LC-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 × 25 cm) eluted at 1 ml per min with 0.1 v/v % TFA and an acetonitrile gradient of 15-45 v/v % over 30 min while acquiring ESI-MS in the positive mode.

Synthesis and Characterization of PEGylated Polyacridine Peptides

PEGylation of the Cys residue on $(Acr\text{-}Lys_n)_3$-Acr-Lys-Cys (where n = 1-6) was achieved by reacting 1 μmol of peptide with 1.1 μmol of PEG_{5kDa}–maleimide in 4 ml of 100 mM Hepesbuffer pH 7 for 12 hrs at RT. PEGylated peptides were purified by semi-preparative RP-HPLC as 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 acetate salt form. The major peak corresponding to the PEG-peptide eluted in the void volume (100 ml) was pooled, concentrated by rotary evaporation, and freeze-dried. PEG-peptides were

reconstituted in water and quantified by Abs_{409nm} (each acridine $\varepsilon_{409\ nm} = 9266\ M^{-1}\ cm^{-1}$) to determine isolated yield (Table 1). PEG-peptides were characterized by MALDI-TOF MS by combining 1 nmol with 10 μl of 2 mg per ml α-cyano-4-hydroxycinnamic acid (CHCA) in 50 v/v % acetonitrile and 0.1 v/v % TFA. Samples were spotted onto the target and ionized on a Bruker Biflex III Mass Spectrometer operated in the positive ion mode.

Formulation and Characterization of PEGylated Polyacridine Peptide Polyplexes

The relative binding affinity of PEGylated polyacridine peptides for pGL3 was determined by a fluorophore exclusion assay[37]. pGL3 (200 μl of 5 μg/ml in 5 mM Hepes pH 7.5 containing 0.1 μM thiazole orange) was combined with 0, 0.05, 0.1, 0.13, 0.15, 0.18, 0.2, 0.25, 0.3, 0.4 0.5, or 1 nmol of PEGylated polyacridine peptide in 300 μl of Hepes and allowed to bind at RT for 30 min. Thiazole orange fluorescence was measured using an LS50B fluorometer (Perkin-Elmer, U.K.) 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 mM Hepes pH 7.5 at a pGL3 concentration of 30 μg per ml and a PEGylated polyacridine peptide stoichiometry of 0.8 nmol per μg of pGL3. 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, NY). The zeta potential was determined as the mean of ten measurements immediately following acquisition of the particle size.

Pharmacokinetic Analysis of PEGylated Polyacridine Polyplexes

Radioiodinated pGL3 was prepared as previously described [38]. Triplicate mice were anesthetized by i.p. injection of ketamine hydrochloride (100 mg per kg) and xylazine hydrochloride (10 mg per kg), then underwent a dual cannulation of the right and left jugular veins. An i.v. dose of ^{125}I-pGL3 (3 μg, 1.2 μCi in 50 μl of Hepes buffered mannitol **(**HBM, 5 mM Hepes, 0.27 M mannitol, pH 7.4) or ^{125}I-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 hrs and polyplexes were extracted with 500 μl of phenol/chloroform/isoamyl alcohol (24:25:1) to remove PEGylated peptides, followed by precipitation of DNA with the addition of 1 ml of ethanol[8,15]. The precipitate was collected by centrifugation at 13,000 x 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 TBE buffer at 70 V for 60 min[19]. The gel was dried on a zeta probe membrane and autoradiographed on a Phosphor Imager (Molecular Devices, Sunnyvale CA) following a 15 hr exposure.

Biodistribution Analysis of PEGylated Polyacridine Polyplexes

^{125}I-pGL3 (1.5 μg in 50 μL of HBM, 0.6 μCi) or ^{125}I-pGL3 polyplexes (1.5 μg) were dosed in triplicate mice in the tail vein. At times ranging from 5 min to 6 hrs, mice were anesthetized by i.p. 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, 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 percent of the dose in the organ.

Direct Hydrodynamic (HD) Dosing and HD-Stimulated Expression

pGL3 (1 μg), pGL3 PEGylated polyacridine polyplexes (0.8 nmol per μg of pGL3), pGL3 PEI (N:P of 5) or chitosan (N:P 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 mouse's body weight (1.8 – 2.25 ml based on 20-25 g mice). pGL3, polyplexes or lipoplex were directly hydrodynamically (HD) dosed by administering the 1.8-2.25 ml volume to the tail vein of triplicate mice in 5 sec according to a published procedure[12,39].

HD-stimulated expression was performed by tail vein dosing triplicate mice with 1 μg of pGL3, PEGylated polyacridine polyplexes, PEI polyplex, chitosan polyplex, or Lipofectamine lipoplex in 50 μl of HBM. At times ranging from 5 min to 9 hrs, a HD-stimulatory dose of normal saline (9 wt/vol% of the body weight) was administered over 5 sec. At 24 hrs post HD-stimulation, mice were anesthetized by 3% isofluorane, then administered an i.p. dose of 80 μl (2.4 mg) of D-luciferin (30 μg/μl in phosphate-buffered saline). At 5 min following the D-luciferin dose, mice were imaged for bioluminescence (BLI) on an IVIS Imaging 200 Series (Xenogen). BLI was performed in a light-tight chamber on a temperature-controlled, adjustable stage while isofluorane 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 sec acquisition time. The Xenogen system reported bioluminescence as photons/sec/cm^2/seradian in a 2.86 cm diameter region of interest covering the liver. The integration area was transformed to pmols of luciferase in the liver using a previously reported standard curve[14]. Results were determined to be statistically significant ($p \leq 0.05$) based on a two-tailed unpaired t-test.

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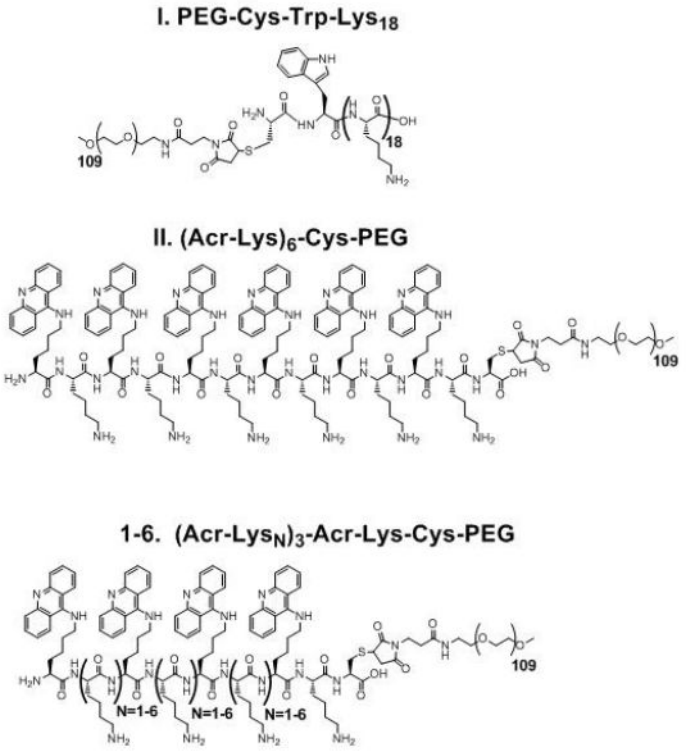

Figure 1. Structures of PEGylated Polyacridine Peptides

The structure of PEGylated polyacridine peptides **1-6** possessing four Lys-ε-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 D-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 ε-amine of L-Lys residues in peptide **4**.

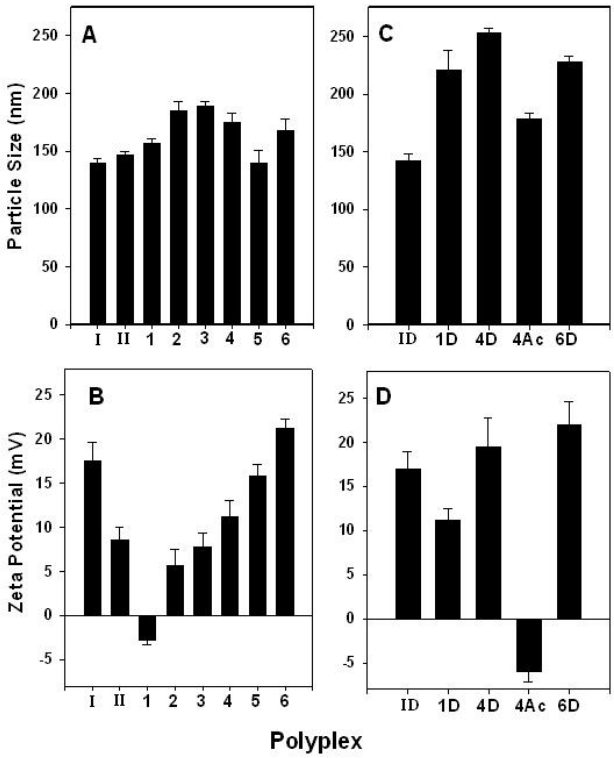

Figure 2. Size and Charge of PEGylated Polyacridine Peptide Polyplexes

Panel A and 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 panel C and D. The results establish the equivalent size and increasing charge for **1**, **2**, **3**, **4**, **5**, and **6** polyplexes, and identified some differences between D versus L peptide polyplexes. The data is plotted as the mean and standard deviation of multiple determinations.

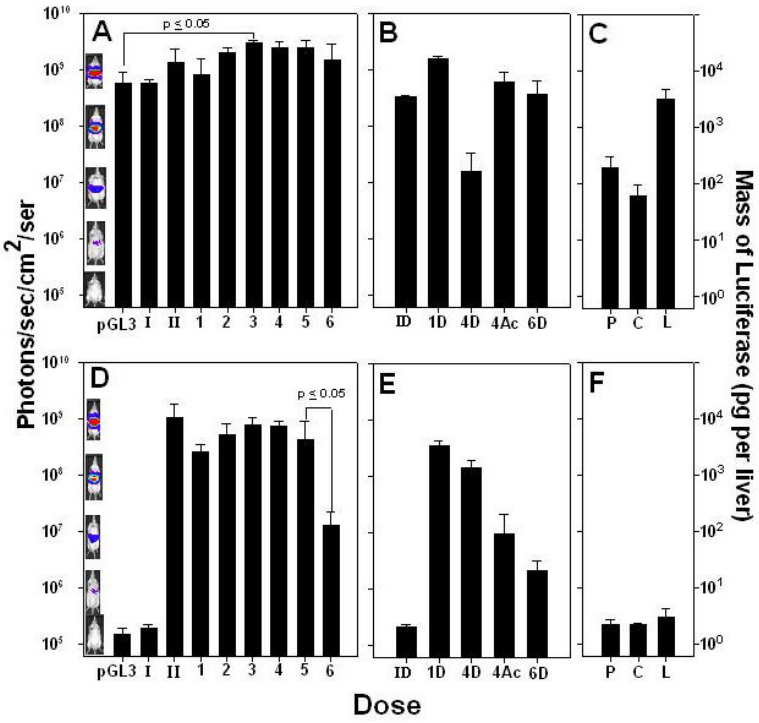

Figure 3. Direct-HD Dosing and HD-Stimulated Expression of PEGylated Polyacridine Peptide Polyplexes

Panel A-C compare the result of direct-HD dosing with panels D-F, which used HD-stimulated dosing (5 min delay) for different gene formulations. Direct-HD dosing was used to administer 1 μg of pGL3 or pGL3 polyplex prepared with 0.8 nmols of PEGylated peptides **I**, **II, 1, 2, 3, 4, 5,** or **6** followed by BLI at 24 hrs to determine the expression of luciferase. Panel B compared control PEGylated peptide (**ID**, **1D**, **4D**, **4Ac** and **6D)** polyplexes administered by direct-HD dosing. PEI (**P**) (5:1 N:P) and chitosan (**C**) (N:P 20:1) polyplexes (1 μg of pGL3) were administered by direct-HD dosing, along with Lipofectamine (**L**) (2:1 Lipid:DNA wt ratio) lipoplexes (1 μg of pGL3) (Panel C). The results establish that, relative to pGL3, PEGylated peptide polyplexes are fully transfection competent when administered by direct-HD. Panels D-F compare the level of luciferase expression when administering 1 μg of pGL3 polyplex or lipoplex (each in 50 μl) by tail vein followed by HD-stimulation with 1.8-2.25 ml of saline after a 5 min delay and measurement of luciferase at 24 hrs. The results establish the complete loss of activity for **pGL3**, **I**, **ID**, **P**, **C** and **L** which is likely due to rapid shedding of the carrier and DNAse metabolism and/or aberrant biodistribution. The results represent the mean and standard deviation of triplicate mice.

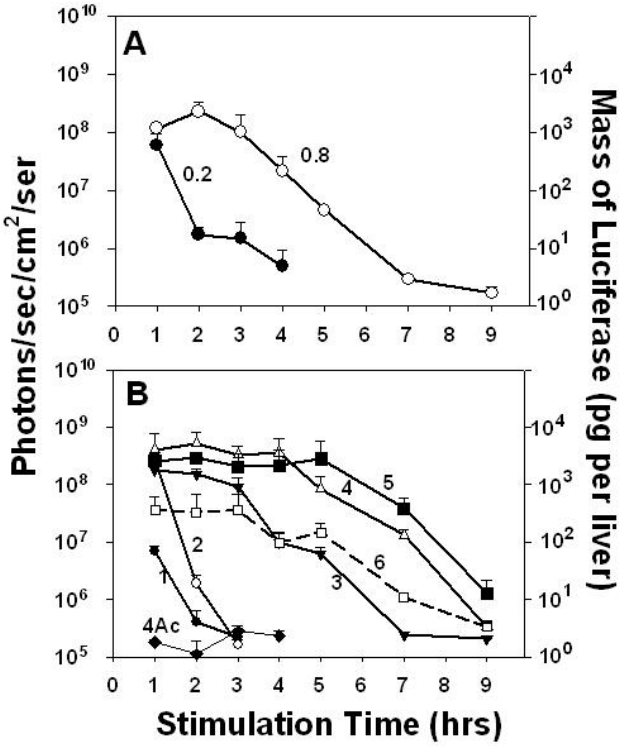

Figure 4. Extended HD-Stimulated Expression of PEGylated Polyacridine Peptide Polyplexes

Panel A illustrates the influence of stoichiometry (0.2 versus 0.8 nmol of **II** per μg of pGL3) on the level of HD-stimulated gene expression with a delay time ranging from 1 to 9 hrs. The results indicate that polyplexes prepared with saturating amounts of **II** remain fully active in the circulation for up to 3 hrs. Panel B compares the level of HD-stimulated gene expression at a constant stoichiometry of 0.8 nmol of peptide per μg of pGL3 for polyplexes prepared with **1**, **2**, **3**, **4**, **5**, **6** and **4Ac** with a delay time ranging from 1 to 9 hrs. The results establish peptide **4** and **5** polyplexes are stable in the circulation for up to 4-5 hrs. All pGL3 polyplex doses were 1 μg (50 μl) administered via the tail vein, followed by HD-stimulation with tail vein administration of 1.8-2.25 ml of saline at times indicated and BLI measurements performed 24 hrs post HD-stimulation. The results represent the mean and standard deviation of triplicate mice.

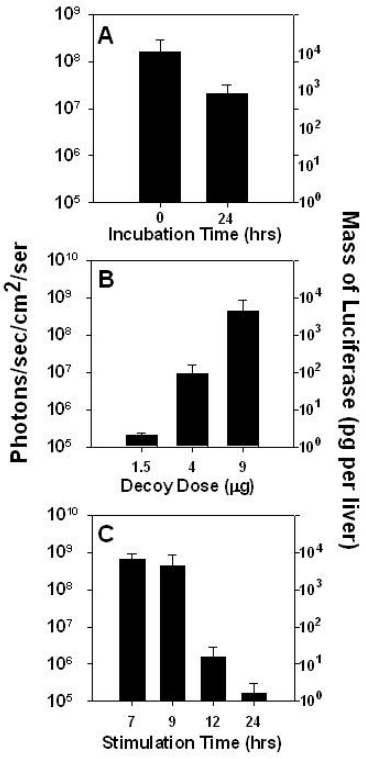

Figure 5. Metabolic Stability and Decoy-Enhanced, HD-Stimulated Gene Expression

Panel A illustrates the result of incubation of pGL3 (1 μg) and 0.8 nmol of **4** in 70 μl of freshly harvested heparinized (5 μl of 50 U per ml) mouse blood at 37°C for 0 or 24 hrs. The polyplex prepared in blood was dosed tail vein in triplicate mice, followed by HD-stimulation at 1 hr and measurement of luciferase expression by BLI at 24 hrs. The results establish that pGL3 peptide **4** polyplexes are deactivated by 90% when incubated in blood for 24 hrs. Panel B illustrates the level of gene expression resulting from tail vein co-administration of 1 μg of pGL3 peptide **4** polyplex delivered with 1.5, 4 or 9 μg of pSEAP peptide **4** polyplex (decoy), followed by HD-stimulation at 9 hr post-pGL3 delivery. The results establish full recovery of expression at 9 hrs when administering 9 μg of decoy polyplex. Panel C illustrates the level of luciferase expression by varying the HD-stimulation delay time from 7 to 24 hrs following tail vein co-administration 1 μg of pGL3 peptide **4** polyplex with 9 μg of decoy. The results represent the means and standard deviation of triplicate mice.

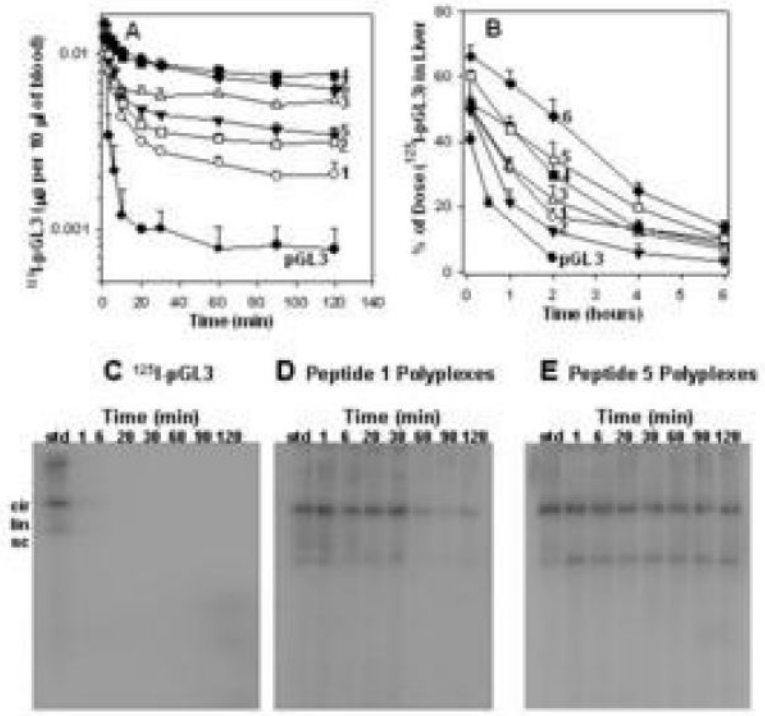

Figure 6. Pharmacokinetics and Biodistribution of PEGylated Polyacridine Peptide Polyplexes

Panel A illustrates the pharmacokinetic profile for ^{125}I-pGL3 and ^{125}I-pGL3 polyplexes prepared with peptides **1**, **2**, **3**, **4**, **5** and **6**. The rapid loss of ^{125}I-pGL3 and ^{125}I-pGL3 peptide **1** polyplexes is due to metabolism as reported previously[8,15,21,28]. The pharmacokinetic profile for peptide **2**-**6** polyplexes is consistent with their DNAse stability in the circulation. The pharmacokinetic time points from panel A were analyzed by agarose gel electrophoresis and autoradiography for DNA (panel C), peptide **1** polyplexes (panel D) and peptide **5** polyplexes (panel E). Panel B illustrates the biodistribution of ^{125}I-pGL3 and ^{125}I-pGL3 polyplexes in liver at times ranging from 5 min to 6 hrs. The results represent the mean and standard deviation of triplicate mice. The derived pharmacokinetic parameters are presented in Table 2, and the complete tissue biodistribution results are included in Table 3.

Table 1

PEGylated Polyacridine Peptide Yields, Masses and Binding Equivalents to DNA

Polyacridine Peptide and PEGylated Polyacridine Peptide Structure	Synthetic Yield (%)	Mass (m/z) Calc/Obs	Binding Equivalent nmol/μg of DNA
$(Acr\text{-}Lys)_3\text{-}Acr\text{-}Lys\text{-}Cys^a$	26	1855.3 / 1855.6	
1. $(Acr\text{-}Lys)_3\text{-}Acr\text{-}Lys\text{-}Cys\text{-}PEG^b$	55	7355 / 7218	0.4
$(Acr\text{-}DLys)_3\text{-}Acr\text{-}DLys\text{-}Cys^a$	26	1855.3 / 1855.8	
1D. $(Acr\text{-}DLys)_3\text{-}Acr\text{-}DLys\text{-}Cys\text{-}PEG^b$	61	7755 / 7500	0.4
$(Acr\text{-}Lys_2)_3\text{-}Acr\text{-}Lys\text{-}Cys^a$	10	2239.9 / 2239.6	
2. $(Acr\text{-}Lys_2)_3\text{-}Acr\text{-}Lys\text{-}Cys\text{-}PEG^b$	42	7740 / 7684	0.18
$(Acr\text{-}Lys_3)_3\text{-}Acr\text{-}Lys\text{-}Cys^a$	19	2624.4 / 2624.6	
3. $(Acr\text{-}Lys_3)_3\text{-}Acr\text{-}Lys\text{-}Cys\text{-}PEG^b$	69	8524 / 8316	0.18
$(Acr\text{-}DLys_4)_3\text{-}Acr\text{-}DLys\text{-}Cys^a$	26	3008.9 / 3008.6	
4D. $(Acr\text{-}DLys_4)_3\text{-}Acr\text{-}DLys\text{-}Cys\text{-}PEG^b$	56	8909 / 8787	0.2
$(Acr\text{-}AcLys_4)_3\text{-}Acr\text{-}AcLys\text{-}Cys^a$	4	3513.3 / 3512.8	
4Ac. $(Acr\text{-}AcLys_4)_3\text{-}Acr\text{-}AcLys\text{-}Cys\text{-}PEG^b$	29	9413 / 9131	0.4
$(Acr\text{-}Lys_5)_3\text{-}Acr\text{-}Lys\text{-}Cys^a$	19	3393.4 / 3393.4	
5. $(Acr\text{-}Lys_5)_3\text{-}Acr\text{-}Lys\text{-}Cys\text{-}PEG^b$	73	9293 / 9202	0.15
$(Acr\text{-}Lys_6)_3\text{-}Acr\text{-}Lys\text{-}Cys^a$	18	3777.9 / 3777.6	
6. $(Acr\text{-}Lys_6)_3\text{-}Acr\text{-}Lys\text{-}Cys\text{-}PEG^b$	56	9678 / 9332	0.13
$(Acr\text{-}DLys_6)_3\text{-}Acr\text{-}DLys\text{-}Cys^a$	13	3777.9 / 3777.4	
6D. $(Acr\text{-}DLys_6)_3\text{-}Acr\text{-}DLys\text{-}Cys\text{-}PEG^b$	47	9678 / 9482	0.2

[a] Mass determined by positive mode ESI-MS.

[b] Mass determined by positive mode MALDI-TOF.

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Table 2

PEGylated Polyacridine Peptide Polyplex 616 Pharmacokinetics[a]

PEGylated Polyacridine Peptide Polyplex	$t_{1/2}\alpha$[b] $(min)^{-1}$	$t_{1/2}\beta$[c] $(min)^{-1}$	V_d[d] (ml)	Cl[e] (ml/min)	MRT[f] (min)	AUC[g] (μg*min/ml)
1. (Acr-Lys)$_3$-Acr-Lys-Cys-PEG / ^{125}I-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 / ^{125}I-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 / ^{125}I-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 / ^{125}I-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 / ^{125}I-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 / ^{125}I-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

[a] Calculated using blood cpm values over 120 min, assuming complete DNA stability.

[b] Calculated α-half-life.

[c] Calculated β-half-life.

[d] Volume of distribution.

[e] Total body clearance rate.

[f] Mean residence time.

[g] Area under the curve.

Table 3

Biodistribution of PEGylated Polyacridine 625 Peptide Polyplexes

	Time (min)	Blood[a]	Liver[b]	Lung[b]	Spleen[b]	Stomach[b]	Kidney[b]	Heart[b]	Large Intestine[b]	Small Intestine[b]	Total[c]
^{125}I-pGL$_3$	5	10.8± 4.3	65.7±2.5	6.1±2.2	2.8±0.3	0.2±0.0	0.9±0.2	0.1±0.0	0.2±0.1	0.4±0.1	81.1±9.7
	60	3.9±1.2	12.7±2.0	0.9±0.2	1.3±0.2	4.5±1.0	3.5±0.6	0.2±0.0	1.1±0.2	2.1±0.3	30.2± 5.8
	120	3.9±1.5	6.1±1.5	0.5±0.2	0.8±0.2	9.4±3.2	1.6±0.9	0.1±0.1	1.9±0.8	2.6±0.5	26.9±8.4
1. (Acr-Lys)$_3$-Acr-Lys-Cys-PEG / ^{125}I-pGL3	5	30.0±3.6	49.9±3.3	2.2±0.6	8.5±2.2	0.5±0.1	0.5±0.2	0.3±0.1	0.6±0.1	0.6±0.3	93.0±10.7
	60	11.4±2.1	31.6±2.9	1.3±0.1	9.1±3.4	4.4±1.6	1.2±0.1	0.2±0.1	1.8±0.5	0.6±0.2	61.6±10.4
	120	9.9±1.3	16.9±2.7	0.8±0.2	8.3±5.1	7.9±3.4	1.6±0.2	0.1±0.0	2.3±0.6	0.8±0.2	48.6±13.7
2. (Acr-Lys$_2$)$_3$-Acr-Lys-Cys-PEG / ^{125}I-pGL3	5	42.4±3.8	49.6±4.3	1.2±0.2	11.6±2.0	0.5±0.2	1.6±0.1	0.3±0.1	0.8±0.1	0.3±0.1	108.0±10.9
	60	32.6±4.4	21.1±4.0	1.2±0.2	10.1±2.5	5.6±3.7	2.0±0.4	0.2±0.0	2.4±0.2	1.0±0.3	76.3±15.9
	120	29.7±2.4	12.5±1.3	0.9±0.2	11.6±6.3	7.4±3.4	1.7±0.1	0.2±0.1	2.5±0.3	1.2±0.2	67.9±14.3
3. (Acr-Lys$_3$)$_3$-Acr-Lys-Cys-PEG / ^{125}I-pGL3	5	41.1±4.3	50.9±7.8	5.8±1.8	7.2±2.1	0.5±0.2	0.9±0.5	0.2±0.1	0.6±0.2	0.5±0.4	107.6±17.6
	60	34.2±2.8	32.3±2.5	6.1±0.2	9.2±2.1	3.3±1.1	2.1±0.4	0.2±0.0	2.5±0.8	0.8±0.4	90.7±10.3
	120	32.1±3.7	22.0±4.8	5.6±1.5	10.5±3.1	4.5±1.1	1.9±0.4	0.2±0.0	2.4±0.5	1.6±0.3	80.6±15.4
4. (Acr-Lys$_4$)$_3$-Acr-Lys-Cys-PEG / ^{125}I-pGL3	5	47.9±5.3	51.8±7.5	2.6±1.8	4.3±0.8	0.4±0.2	1.0±0.5	0.3±0.1	0.4±0.1	0.2±0.1	108.3±16.6
	60	35.9±3.5	44.1±2.0	0.8±0.2	8.6±0.8	2.4±1.1	1.2±0.1	0.2±0.0	1.2±0.2	0.4±0.1	94.9± 8.1
	120	32.0±2.8	29.6±4.8	0.8±0.1	6.5±0.6	4.3±0.8	1.7±0.6	0.2±0.1	1.6±0.6	0.9±0.1	77.5±10.5
5. (Acr-Lys$_5$)$_3$-Acr-Lys-Cys-PEG / ^{125}I-pGL3	5	41.3±3.4	60.2±2.5	2.3±0.0	3.0±0.8	0.8±0.1	1.3±0.3	0.5±0.1	0.5±0.1	0.2±0.0	110.2±7.5
	60	24.1±3.5	43.7±4.0	1.4±0.7	6.3±1.5	3.0±1.7	2.1±0.5	0.2±0.0	2.0±0.6	0.5±0.1	83.3±12.6
	120	20.9±3.0	34.7±5.3	1.0±0.2	3.0±1.3	5.9±0.8	1.9±0.9	0.2±0.0	2.8±0.8	0.8±0.0	78.1±12.1
6. (Acr-Lys$_6$)$_3$-Acr-Lys-Cys-PEG / ^{125}I-pGL3	5	40.7±3.6	66.2±3.3	4.4±0.9	1.4±0.7	0.3±0.1	1.4±0.1	0.5±0.2	0.7±0.1	0.3±0.1	115.9±9.1
	60	37.4±3.7	57.8±4.1	2.9±0.2	5.3±0.4	3.5±0.7	1.2±0.1	0.4±0.0	1.1±0.2	0.7±0.0	110.5±9.6
	120	31.5±2.9	47.8±5.2	2.1±1.1	3.1±0.6	8.1±0.2	0.8±0.1	0.2±0.1	1.3±0.3	0.6±0.1	96.6±10.5

[a] Percent of dose based on pharmacokinetic analysis, assuming a total mouse blood volume of 1.5 ml

[b] Percent of dose based on gamma counting of tissue.

[c] Total percent of dose recovered.