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Discovery of Metabolically Stabilized Electronegative Polyacridine-PEG Peptide DNA Open Polyplexes

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Abstract

Cationic condensing peptides and polymers bind electrostatically to DNA to form cationic polyplexes. While many cationic polyplexes are able to achieve in vitro transfection mediated through electrostatic interactions, few have been able to mediate gene transfer in vivo. The present study describes the development and testing of polyacridine PEG-peptides that bind to plasmid DNA by intercalation resulting in electronegative open polyplex DNA. Polyacridine PEG-peptides were prepared by chemically conjugating 6-(9-acridinylamino) hexanoic acid onto side chains of Lys in PEG-Cys-Trp-(Lys)_{3, 4, or 5}. The resulting PEG-Cys-Trp-(Lys-(Acr))_{3, 4, or 5} peptides bound tightly to DNA by polyintercalation, rather than electrostatic binding. Unlike polycationic polyplexes, polyacridine PEG-peptide polyplexes were anionic and open coiled, as revealed by zeta potential and atomic force microscopy. PEG-Cys-Trp-(Lys-(Acr))₅ showed the highest DNA binding affinity and the greatest ability to protect DNA from metabolism by DNase. Polyacridine PEG-peptide DNA open polyplexes were dosed intramuscularly and electroporated in mice to demonstrate their functional activity in gene transfer. These results establish polyacridine PEG-peptide DNA open polyplexes as a novel gene delivery method for in vivo use.

Introduction

Nonviral gene delivery systems have traditionally relied on reversible binding between cationic carriers and anionic oligonucleotides (1–3). The resulting lipoplexes and polyplexes typically possess a positive surface charge as determined by zeta potential measurements. While cationic DNA lipoplexes and polyplexes have shown the ability to mediate in vitro gene transfer through electrostatic binding to cell surfaces, they exhibit poor gene transfer properties in vivo, in part due to their overall cationic charge (4,5). There have been many attempts to mask the positive charge of cationic polyplexes with polyethylene glycol (PEG) to improve blood compatibility (6–11).

Alternatively, it is difficult to prepare an electronegative DNA polyplex using cationic polymers. This has been attempted by either partially titrating a polycationic polymer with plasmid DNA or by using a layer-by-layer addition of oppositely charge polymers in an attempt to reverse the charge of polyplexes (12–14). The resulting electronegative polyplexes are formed in a delicate and unstable equilibrium (15). Attempting to reverse the charge of a cationic polyplex with the addition of an anionic polymer can lead to dissociation of the DNA. Similarly, if titrated to below the charge equivalency point, the polyplex is not completely protected against DNase, whereas if titrated to charge equivalency, the resulting neutral polyplexes are hydrophobic and aggregate. Titration past the equivalency point, results in collapse of a polyplex into a cationic colloidal particle that

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is relatively stable but highly cationic and thereby less compatible with blood components (5). Consequently, we sought to find an alternative approach to ionic binding to DNA that would result in stable electronegative polyplexes.

In principle, the high affinity DNA binding achieved by polyintercalation could result in metabolically stable electronegative polyplexes (16). Electronegative polyplexes may bind to fewer proteins and thereby be more blood and tissue compatible to allow delivery via intramuscle electroporation (IM-EP) (17–20).

Polyacridine containing polymers have been previously investigated as gene transfer agents by Szoka, Vierling and Neilsen (21–23). Their studies utilized polyacridine polymers possessing either one, two or three acridine units conjugated to either a neoglycopeptide or a nuclear localizing sequence. While these polyacridine carriers could bind to DNA, they possessed modest gene transfer activity in vitro (22,23). Alternatively, we have recently reported that a polyacridine peptide modified with a melittin fusogenic peptide is a potent in vitro gene transfer agent (24). The most potent polyacridine-melittin gene delivery peptide contained four acridines and possessed a sequence of Lys(Acr)-Arg-Lys(Acr)-Arg-Lys(Acr)-Arg-Lys(Acr)-Arg-Cys joined to melittin by a disulfide bond. Polyacridine-melittin bioconjugates formed electropositive DNA polyplexes in which their potency was dependent upon the number of acridines, the identity of the spacing amino acid, and the presence of a reducible disulfide bond between polyacridine and melittin.

To date, there are no reports of polyacridine peptides modified with PEG. The present study demonstrates a synthetic strategy to modify the side chains of Lys in PEG-Cys-Trp-(Lys)_n peptides with acridine. The resulting PEG-Cys-Trp-(Lys(Acr))_n peptides bind reversibly to DNA through intercalation and demonstrate the ability to form a unique electronegative open polyplex DNA structure that is protected from DNase and maintains transfection competency in vivo. The unique properties of PEGylated polyacridine DNA electronegative open polyplexes afford desirable characteristics that are more compatible with in vivo nonviral gene delivery compared to cationic polyplexes.

Materials and Methods

N-terminal Fmoc protected amino acids, 9-hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC), and Wang resin were purchased from Advanced ChemTech (Lexington, KY). Sephadex G-25, HEPES buffer, tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), diisopropylethylamine (DIPEA), piperidine, acetic anhydride, triisopropylsilane (TIS), DNase I (EC 3.1.21.1), 9-chloroacridine, and thiazole orange were obtained from Sigma Chemical Co (St. Louis, MO). Acetonitrile, N,N-Dimethylformamide (DMF), and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Pittsburgh, PA). Agarose was obtained from Gibco-BRL. mPEG-maleimide 5000 Da was purchased from Nektar (Huntsville, AL), mPEG-amine (5,000 Da) was purchased from Creative Biotechnology (Winston-Salem, NC), and bis-amino PEG (2,000 Da) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). The 5.3 kb luciferase plasmid, pGL3 control vector, containing a SV40 promoter and enhancer was obtained from Promega (Madison, WI). Plasmid DNA was amplified in DH5α strain of *E. coli* and purified according to the manufacturer's instructions.

Synthesis and Characterization of PEGylated Cys-Trp-Lys_n Peptides

Cys-Trp-Lys_n synthesis was carried out using standard Fmoc chemistry on a computer interfaced Advanced Chemtech APEX 396 solid phase peptide synthesizer with 9-hydroxybenzotriazole and diisopropylcarbodiimide double couplings followed by N-capping with acetic anhydride as described previously (25). Cleavage and deprotection were

accomplished by reacting the resin with TFA: TIS: water (95:2.5:2.5) at RT for 3 hrs. The crude peptide was precipitated in cold diethyl ether, centrifuged, and the supernatant was decanted. Crude peptides were reconstituted in 0.1% TFA and purified on a preparatory RP-HPLC using a Vydac C18 column (2 × 25 cm) eluted at 10 ml/min with 0.1 v/v% TFA and a gradient of acetonitrile of 5–25% over 30 min while monitoring Abs 280 nm. Purified peptides were reconstituted in 0.1% TFA and quantified by Abs (Trp $\epsilon_{280\text{nm}} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$) to determine the isolated yield of 30%. The purified peptides were characterized using LC-MS by separation on a Vydac C18 analytical column (0.47 × 25 cm) eluted at 0.7 ml/min with 0.1% TFA and a gradient of acetonitrile of 5–25% over 30 min while detecting ESI-MS in the positive ion mode.

PEGylation of the Cys residue on Cys-Trp-Lys_{3, 4} and 5 was achieved by reacting 0.9 mol equivalents of peptide with 1 mol equivalent of PEG₅₀₀₀ Da-maleimide in 100 mM sodium phosphate buffer pH 7 for 1 hr at RT (25). PEGylated peptides were purified on a G-25 column (2.5 × 50 cm) eluted with 0.1% acetic acid while monitoring Abs 280 nm. The peak corresponding to the PEG-peptide eluted in the void volume (100 ml), was pooled, concentrated by rotary evaporation, and freeze-dried. The PEG-peptide was reconstituted in 0.1% TFA, and quantified by Abs (Trp $\epsilon_{280\text{nm}} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$) to determine an isolated yield of 80%. PEG-peptides were characterized by MALDI-TOF MS by combining 1 nmol with α -cyano-4-hydroxycinnamic acid (CHCA) prepared in 50% (v/v) acetonitrile and 0.1% TFA. Samples were spotted onto the target and ionized on a Bruker Biflex III Mass Spectrometer operated in the positive ion mode.

Synthesis of Polyacridine PEG Peptides and Mono and Bis-Acridine PEGs

6-(9-Acridinylamino) hexanoic acid was prepared according to a prior published procedure (26). The carboxyl group on 6-(9-acridinylamino) hexanoic acid was activated with 1.1 mol equivalents of DIC and HOBt in DMF. Activated 6-(9-acridinylamino) hexanoic acid (12 mol equivalents) was reacted with 2.5 μmol of PEG-Cys-Trp-Lys_{3, 4} and 5 in 3 ml of DMF at RT for 24 hrs. The PEG-Cys-Trp-(Lys(Acr))_{3, 4} and 5 peptides were purified using a G-25 column (2.5 × 50 cm) eluted with 0.1 % acetic acid while monitoring Abs 280 nm. The product peak eluting at 100 ml was pooled, freeze dried, and characterized using MALDI-TOF MS as described above. A yield of 42% of PEG-Cys-Trp-(Lys(Acr))_{3, 4} and 5 peptides was determined by acridine Abs ($\epsilon_{409\text{nm}} = 9266 \text{ M}^{-1} \text{ cm}^{-1}$) assuming additivity of acridine molar absorptivity and complete conjugation to the ϵ -amine of Lys. Similarly, mono and bis-acridinylated PEGs were prepared by reacting 6 mol equivalents of activated 6-(9-acridinylamino) hexanoic acid with PEG-amine or bis-amino PEG (2 μmol) in 2 ml of DMF. The acridinylated PEGs were purified by G-25 column and characterized by MALDI-TOF MS.

Thiazole Orange Displacement Assay

The binding of polyacridine PEG-peptides to DNA was determined using an intercalator dye displacement assay (27). pGL3 (1 μg) was prepared in 5 mM Hepes pH 7.4 containing 40 nM thiazole orange. Polyacridine PEG-peptides were added to pGL3 to prepare samples of 0, 0.25, 0.5, 1, 2, 3, and 4 nmol per μg of DNA in a final volume of 500 μL . The fluorescence intensity of each sample was measured using an LS50B fluorometer (Perkin-Elmer, UK) with an excitation wavelength of 500 nm while monitoring emission at 530 nm. The data was converted to percent by comparison to the fluorescence intensity of thiazole orange in buffer (0%) and pGL3 with fully bound thiazole orange (100%).

Gel Band Shift and DNase Protection Assay

pGL3 (1 μg) or DNA polyplexed (1 μg) prepared in 18 μL of normal saline were combined with 1 nmol of either mono-acridine PEG, bis-acridine PEG, PEG-Cys-(Lys-Acr)₃, PEG-

Cys-(Lys-Acr)₄, or PEG-Cys-(Lys-Acr)₅ and 2 µl of loading buffer (28). The samples were loaded onto a 1% agarose gel (50 ml) and electrophoresed in TAE buffer at 80 V for 90 min. The gel was stained in 2 mg/ml ethidium bromide at 5°C overnight, and the transilluminated gels were photographed using Polaroid 667 film. pGL3 (1 µg) or DNA polyplexes (1 µg) prepared with 1 nmol of either mono-acridine PEG, bis-acridine PEG, PEG-Cys-(Lys-Acr)₃, PEG-Cys-(Lys-Acr)₄, or PEG-Cys-(Lys-Acr)₅ in 20 µl of normal saline was incubated with 0.06 U of DNase I at 37°C for 0–20 min (29). The samples were applied to a 1% agarose gel and electrophoresed as described above.

Particle Size and Zeta Potential of Polyacridine PEG-peptide DNA Open Polyplexes

Polyacridine PEG-peptide DNA polyplexes were formed by combining 10 µg of pGL3 (in 500 µl of 5 mM Hepes pH 7.4) with an equal volume containing either 1, 2, 4, 8, 12, 16, or 20 nmol of polyacridine PEG-peptide while vortexing. The polyplexes were equilibrated at RT for 30 min prior to analysis of particle size (QELS) and zeta potential using a Brookhaven ZetaPlus (Brookhaven Instruments, Holtsville, NY). The particle size diameter and zeta potential are reported as the mean and standard deviation of ten measurements.

Atomic Force Microscopic Analysis of Polyacridine PEG-peptide DNA Open Polyplexes

pGL3 was prepared at a concentration of 100 µg per ml in 10 mM Tris containing 1 mM EDTA pH 8. DNA polyplexes were prepared identically with the addition of PEG-Cys-Trp-(Lys(Acr))₅ at either 0.2 or 1 nmol per µg of DNA. pGL3 and DNA polyplexes were diluted 10-fold in 40 mM Hepes containing 5 mM nickel chloride pH 6.7, then deposited on a fresh cleaved mica surface and incubated for 10 min, followed by washing with deionized water (30). Alternatively, polyacridine-melittin DNA polyplexes were prepared at 0.5 nmol of peptide per µg of DNA at a concentration of 100 µg per ml of DNA and directly deposited on a freshly cleaved mica surface and allowed to bind for 10 min prior to washing with deionized water (24). Images were captured using an Asylum atomic force microscope (AFM) MFP3D (Santa Barbara, CA) operated in the AC-mode using a silicon cantilever (Ultrasharp NSC15/AIBS, Mikro Masch).

Intramuscle-Electroporation Dosing

Male ICR mice (Harlan), weighing 25–30 g were prepared for IM-EP dosing by anesthetization with an intraperitoneal dose of 200 µl of ketamine/xylazine (20 mg/ml and 2 mg/ml, respectively). The hamstring muscles were sheared and swabbed with 70% ethanol prior to administering a 50 µl dose over 10 sec in normal saline to both gastrocnemius muscles in 2 mice by a 1 ml monoject syringe (1cc, 28 G × ½). After dosing the BTX 2-Needle Array Electrode was inserted into the skin with the electrodes straddling the dosing site. Successive electronic stimulation was generated by the EMC BTX 830, Square-wave Pulse Generator (BTX, Harvard Apparatus) as the power source. At 1 min post-administration of the DNA dose, the pulse generator delivered six successive 100 V pulses over 20 msec with a 100 msec interval between pulses (31).

Bioluminescence Imaging

Luciferase reporter gene expression was quantified by bioluminescence imaging (BLI) at 2–14 days following IM-EP using an IVIS Imaging System 200 Series (Xenogen) (32). Mice were anesthetized by isofluorane (2% flow with oxygen) and dosed intramuscularly with 40 µL of 30 mg per ml D-Luciferin (GoldBio). Bioluminescent images were acquired 5 min post substrate administration and acquired for 1 min with a 24.6 cm field of view. The resultant grayscale images with a colormap overlay were analyzed using the IgorPro 4.09 software (LivingImage). Luciferase expression is reported as photons/sec/cm²/steradian within a uniformly defined region of interest.

Results

Several polyintercalator constructs that bind to double stranded DNA have been reported in prior studies aimed at delivering plasmid DNA (21–23). These studies designed and tested delivery molecules possessing one-three acridine dyes displayed on a branched polymer modified with a neoglycopeptide or a nuclear localizing peptide, the later of which were shown to mediate in vitro gene expression (22,23). These studies established that two to three acridines was required to achieved sufficient DNA binding affinity for in vitro transfection.

In the present study we sought to use a synthetic strategy that would allow for the incorporation of more than three acridines by covalent modification of the side chain of PEGylated polylysine. This was accomplished by first modifying acridine to possess a 6-amino hexanoic acid and then activating the carboxyl group to allow coupling to the ϵ -amines of PEG-Cys-Trp-(Lys)₃, 4, and 5 to afford PEG-Cys-Trp-(Lys(Acr))₃, 4 and 5 (Fig. 1). In addition, conjugation of acridine 6-amino hexanoic acid directly to PEG-amine and bis amino PEG afforded constructs with either one or two acridines (Fig. 1). This strategy provided an advantage by allowing the comparison of DNA binding affinity with PEGs modified with 1–5 acridines.

Chromatographic evidence supporting the synthesis of PEG-Cys-Trp-(Lys(Acr))₅ is illustrated in figure 2. Modification of Cys-Trp-(Lys)₅ with PEG results in a significant delay in elution on RP-HPLC compared to Cys-Trp-(Lys)₅. However, upon conjugation of five acridines to PEG-Cys-Trp-(Lys)₅, the resulting PEG-Cys-Trp-(Lys(Acr))₅ elutes only slightly later, and is detected at Abs 409 nm.

The complete conjugation of acridine to the each Lys side chain was established using MALDITOF MS analysis. Conjugation of Cys-Trp-(Lys)₅ (948.8 g/mol) with polydisperse PEG of average mass of 5425 Da results in a PEG-Cys-Trp-(Lys)₅ with an apparent average mass of 6546 Da (Fig. 3A–C). Purified PEG-Cys-Trp-(Lys(Acr))₅ produced an average mass of 7853 Da (Fig. 3D), consistent with the conjugation of 4.4 acridines. Similar mass spectral results were obtained when preparing PEG-Cys-Trp-(Lys(Acr))₃ and PEG-Cys-Trp-(Lys(Acr))₄ as summarized in Table 1.

The relative DNA binding affinity of mono and bis-acridine PEG compared to polyacridine PEG-peptides was established using a thiazole orange displacement assay (33) (Fig 4A). Titration of 0.2–4 nmols of mono-acridine PEG with 1 μ g of pGL3 resulted in minimal displacement of thiazole orange. By comparison, bis-acridine PEG demonstrated higher affinity, resulting in a decrease in fluorescence intensity by approximately 40% at 1 nmol per μ g of DNA. The apparent DNA binding affinity of PEG-Cys-Trp-(Lys(Acr))_{3–5} was higher than either mono or bis-acridine PEG, resulting in approximately 10% fluorescence intensity at a stoichiometry of 1 nmol per μ g of DNA (Fig. 4A).

The DNA binding affinity was also compared by a band shift assay performed at a constant stoichiometry of 1 nmol per μ g of DNA (Fig. 4B). Mono-acridine PEG failed to produce a band shift relative to plasmid DNA (Fig. 4B lane 1 and 2), whereas bis-acridine PEG partially retarded the migration of DNA (Fig. 4B lane 3). PEG-Cys-Trp-(Lys(Acr))₃ and 4 both further retarded DNA migration to a similar extent (lanes 4 and 5), whereas PEG-Cys-Trp-(Lys(Acr))₅ led to complete retardation of DNA migration and also inhibited intense ethidium staining (Fig. 4B. Lane 6). Conversely, PEG-Cys-Trp-(Lys)₅ was unable to cause a band DNA shift (not shown).

A further demonstration of polyacridine binding to DNA binding utilized a titration experiment while monitoring both the particle size and zeta potential of DNA polyplexes as

a function of increasing concentration of polyacridine (Fig. 5). The titration of PEG-Cys-Trp-(Lys(Acr))₃ with pGL3 results in a decrease in the apparent particle diameter to approximately 200 nm at 0.2 nmols per μg of DNA but then plateaued at higher stoichiometries. The zeta potential gradually increased from -15 to -5 mV when titrating with 0.1 to 2 nmol of PEG-Cys-Trp-(Lys(Acr))₃ per μg of DNA, indicating that the polyplexes remained electronegative (Fig. 5A). A nearly identical trend was observed when titrating pGL3 with PEG-Cys-Trp-(Lys(Acr))₄ and PEG-Cys-Trp-(Lys(Acr))₅ (Fig. 5B and C), with the exception that the latter reached an asymptote of -5 mV at 1 nmol per μg of DNA due to its greater DNA binding affinity.

The unusual zeta potential properties of the polyacridine DNA polyplexes prompted an investigation into their morphology using atomic force microscopy. Under analysis by AFM, plasmid DNA is observed as an open circular structure when immobilized onto electropositive mica prepared with immobilized Ni (Fig. 6A). By comparison, PEG-Cys-Trp-(Lys(Acr))₅ DNA polyplexes prepared at either 0.2 or 1 nmol per μg of DNA also resulted in an open circular structure, which we term open polyplexes (Fig. 6B and C). Comparison of the size and shape of open polyplexes with plasmid DNA reveals a slightly more compact structure that becomes progressively more tightly wound at higher stoichiometry of polyacridine. The appearance of smaller particles in figures 6B and C are the result of AFM tip irregularities (34). Finer analysis of the data in figures 6B and C suggests that the thickness of the open polyplex DNA is reduced by 20% relative to naked DNA. The open polyplex structure observed by AFM is quite unique when compared to an electropositive polyplex prepared with polyacridine melittin (Fig. 6D).

We conjectured that the DNA in open polyplexes may be protected from metabolic degradation by serum endonucleases. The metabolic stability was evaluated by treating pGL3 with DNase followed by gel electrophoresis. Unprotected pGL3 is significantly degraded by DNase in 5 min and completely degraded by 10 min (Fig 7A, lanes 2 and 3). The weak binding affinity afforded by mono or bis-acridine PEG failed to protect DNA from metabolism (Fig. 7B and C). Comparison of the metabolic stability of PEG-Cys-Trp-(Lys(Acr))₃, 4 and 5 DNA open polyplexes demonstrated that PEG-Cys-Trp-(Lys(Acr))₃ and PEG-Cys-Trp-(Lys(Acr))₄ provided partial protection (Fig. 7D and E), whereas PEG-Cys-Trp-(Lys(Acr))₅ provided complete protection (Fig. 7F). Decreasing the stoichiometry from 1 nmol of PEG-Cys-Trp-(Lys(Acr))₅ to 0.4 or 0.2 nmol per μg of DNA established that at lower stoichiometries the DNA was more susceptible to metabolism (Fig. 7G and H).

The in vivo gene transfer properties of PEG-Cys-(Lys-(Acr))₅-DNA open polyplexes were evaluated in mice following IM-EP. pGL3 and pGL3 PEG-Cys-(Lys-(Acr))₅ open polyplexes prepared at 0.2, 2, and 4 nmol per μg of DNA formulations were administered by IM-EP and the expression was monitored for two weeks by bioluminescence imaging (Fig. 8). At day 2 following dosing, pGL3 and the DNA open polyplexes prepared at 2 nmol per μg of DNA produced identical expression levels. Open polyplexes prepared at 4 nmol per μg of DNA showed approximately 10-fold lower expression, whereas a formulation prepared with 0.2 nmol per μg of DNA resulted in a 100-fold decrease in expression. A similar expression time-course was observed for pGL3 and DNA open polyplexes prepared with 0.2 and 2 nmol of PEG-Cys-(Lys-(Acr))₅ per μg of DNA resulting in a 10-fold decrease over 14 days. The expression time-course for open polyplexes prepared at 4 nmol of PEG-Cys-(Lys-(Acr))₅ per μg of DNA was extended, resulting in only a 2–3 fold loss in expression across the 14 day sampling period.

Discussion

An important function of a gene delivery carrier is to protect DNA from premature metabolism during transit (35). The present study demonstrates that polyintercalation provides strong and reversible binding to DNA that delays metabolic degradation. The main advantage of this approach over polycationic polymer binding to DNA is that high affinity can be achieved with a short polyacridine and that the charge of the resulting DNA polyplexes can be controlled to be either positive or negative, depending on intended applications in vitro or in vivo (24). Prior reports of the cellular toxicity of known intercalators demonstrate a dependency on both chemical structure and dose (36). Little or no information is available regarding the toxicity of PEGylated polyacridine peptides.

To evaluate the relationship between DNA binding affinity and metabolic stability, five DNA carriers were prepared that varied the number of acridines from 1 to 5. The synthetic strategy conjugated 6-(9-acridinylamino) hexanoic acid with the primary amine(s) on PEG-amine, bis-amino PEG, and the ϵ -amines on PEG-Cys-Trp-(Lys)_{3, 4} or ₅. This strategy not only simplified the chemical preparation of the delivery molecules, but also allowed for a long 12 atom tether between each acridine and the peptide backbone to facilitate flexible multivalent binding of acridine with DNA.

The DNA binding affinity significantly increased for PEG-Cys-(Lys-(Acr))_{3, 4}, and ₅ compared to mono and bis-acridine PEG as determined by both fluorophore displacement and DNA band shift on agarose gel electrophoresis (Fig. 4A and 4B). While the QELS particle size suggested the formation of large polyplexes with an apparent diameter of 200 nm, the zeta potential revealed that for PEG-Cys-(Lys-(Acr))_{3, 4}, and ₅ polyplexes were negatively charged. AFM images established that the shape of PEG-Cys-(Lys-(Acr))_{3, 4}, and ₅ DNA polyplexes were open coiled structures that closely resemble plasmid DNA (Fig. 6). The observed electronegative open polyplexes are distinct from electropositive condensed polyplexes that are formed by polycation binding with DNA. The metabolic stability afforded by comparing PEG-Cys-(Lys-(Acr))_{3, 4} and ₅ establish a relationship between the apparent DNA binding affinity and the degree of protection from DNase. Additionally, the metabolic protection of DNA afforded by PEG-Cys-(Lys-(Acr))₅ was concentration dependent.

IM-EP of cationic DNA polyplexes results in lower gene expression levels compared to naked DNA alone (20,37,38). This may be due to the charge of cationic polyplexes that are unable to electromigrate through the transient pore formed during electroporation (20). In contrast, the IM-EP of DNA versus PEG-Cys-(Lys-(Acr))₅ DNA open polyplexes resulted in very similar levels and duration of expression in mice over two weeks. However, at an elevated stoichiometry of PEG-Cys-(Lys-(Acr))₅, a lower but more sustained expression was determined, perhaps due to the metabolic protection afforded by the peptide at higher stoichiometries.

The results of this study establish a new family of PEGylated polyacridine gene delivery carriers that bind to DNA and provide protection against DNase. The unique anionic open polyplex structure is compatible with IM-EP. Given the flexibility of design, polyacridine gene delivery systems could be optimized for a variety of other nonviral gene delivery applications.

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Figure 1. Preparation of PEGylated Polyacridine Bioconjugates

PEG₅₀₀₀ Da-maleimide was reacted with the Cys residue on Cys-Trp-Lys₃, Cys-Trp-Lys₄, and Cys-Trp-Lys₅ to form PEG-Cys-Trp-(Lys)₃, 4, and 5. Activated 6-(9-acridinylamino) hexanoic acid was reacted with ε-amines of Lys to form PEG-Cys-Trp-(Lys(Acr))₃, 4, and 5. Alternatively, mono and bis PEG-amine were reacted with activated 6-(9-acridinylamino) hexanoic acid to prepare mono-Acr-PEG and bis-Acr-PEG.

Figure 2. RP-HPLC Analysis of PEG-Cys-Trp-(Lys-(Acr))₅

Cys-Trp-(Lys)₅ elutes at 10 min on RP-HPLC eluted with 0.1% TFA and an acetonitrile gradient 5–60% over 30 min while detecting Trp by Abs at 280 nm (panel A). The inset shows the ESI-MS of Cys-Trp-Lys₅ with an [M+H] = 982.8 Da. Purified PEG-Cys-Trp-(Lys)₅ elutes at 24 min under identical gradient and detection (panel B). Purified PEG-Cys-Trp-(Lys(Acr))₅ elutes as a broad peak at 25 min and is detected by Abs 409 nm due to acridine (panel C).

Figure 3. Mass Spectral Analysis of PEG-Cys-Trp-(Lys(Acr))₅

MALDI-TOF MS analysis PEG₅₀₀₀ Da-maleimide resulted in an average mass with m/z of 5425 (panel A). MS analysis of PEG-Cys-Trp-(Lys)₅ resulted in a mass shift of 1121 Da, consistent with the formation of the PEG-peptide with m/z of 6546 (panel B). Following conjugation of five 6-(9-acridinylamino) hexanoic acid residues (290 g/mol), the average mass increases by 1307 Da, consistent with the formation of PEG- Cys-Trp-(Lys(Acr))₅ with m/z of 7853 (panel C).

Figure 4. Relative Binding Affinity of Mono-Acridine-PEG, Bis-Acridine-PEG, and Polyacridine Peptides with DNA

The relative binding affinity of mono and bis-acridine-PEG, and PEG-Cys-Trp-(Lys(Acr))₃, 4, and 5 for DNA was determined using a thiazole orange dye displacement assay and agarose gel band shift assay. Thiazole orange displacement established weak DNA binding for mono (●) and bis-acridine-PEG (▼), with higher and indistinguishable affinity determined for PEG-Cys-Trp-(Lys(Acr))₃ (■), 4 (▲), and 5 (◆) (panel A). The DNA binding affinity was also compared by agarose gel electrophoresis DNA migration (1 µg) (lane 1) relative to polyplexes prepared with 1 nmol each of mono-acridine PEG (lane 2), bis-acridine PEG (lane 3), PEG-Cys-(Lys(Acr))₃ (lane 4), PEG-Cys-(Lys(Acr))₄ (lane 5), or PEG-Cys-(Lys(Acr))₅ (lane 6) (Panel B). The results established maximal binding affinity with PEG-Cys-(Lys(Acr))₅ (lane 6).

Figure 5. Particle Size and Zeta Potential of Polyacridine-Peptide DNA Polyplexes

The mean diameter size and zeta potential of DNA polyplexes prepared with 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, or 2 nmol of PEG-Cys-(Lys(Acr))₃, ₄, and ₅ per μg of DNA were compared. At 0.2 nmols of polyacridine peptide or higher, PEG-Cys-(Lys(Acr))₃ formed polyplexes of apparent mean diameter of 200 nm that remained unchanged throughout the titration (Panel A, dashed line). In contrast, the zeta potential progressively increased from -15mV to approximately -2mV when titrating from 0.1–2nmol per μg of DNA (panel A, solid line). Nearly identical results were obtained when titrating with PEG-Cys-(Lys(Acr))₄ (panel B) and PEG-Cys-(Lys(Acr))₅ (panel C).

Figure 6. Atomic Force Microscopy Analysis of Plasmid DNA and Polyacridine DNA Polyplexes
AFM was used to compare the relative morphology of plasmid DNA or PEG-Cys-Trp-(Lys(Acr))₅ DNA polyplexes bound to electropositively charged mica in panels A–C. Plasmid DNA appears as an open circular structure (panel A) of comparable dimensions relative to PEG-Cys-Trp-(Lys(Acr))₅ DNA polyplexes prepared at either 0.2 nmol per μg of DNA (panel B) or 1 nmol per μg of DNA (panel C). Alternatively, a cationic polyplex prepared with polyacridine melittin binds to electronegative mica and appears as a collapsed structure (panel D). The results establish polyacridine PEG peptides bind to DNA to form electronegative open polyplexes that possess similar morphology as plasmid DNA.

Figure 7. Relative Metabolic Stability of Mono-Acridine-PEG, Bis-Acridine-PEG, and Polyacridine Peptides DNA Polyplexes

The relative metabolic stability of DNA polyplexes was compared with plasmid DNA by agarose gel electrophoresis. Plasmid DNA (1 μ g) (panel A), or DNA polyplexes prepared with 1 nmol each of mono-acridine PEG (panel B), bis-acridine PEG (panel C), PEG-Cys-(Lys(Acr))₃ (panel D), PEG-Cys-(Lys(Acr))₄ (panel E), or PEG-Cys-(Lys(Acr))₅ (panel F) were incubated with 0.06 U of DNase at 37°C for 0 (lane 1), 5 (lane 2), 10 (lane 3) and 20 (lane 4) min. PEG-Cys-(Lys(Acr))₅ DNA polyplexes were also prepared at 0.2 (panel G) and 0.4 (panel H) nmol of polyacridine peptide per μ g of DNA and digested with DNase. The results demonstrate that PEG-Cys-(Lys(Acr))₅ provided the greatest protection at 1 nmol per μ g of DNA, (panel F) while the lower stoichiometries of 0.2 or 0.4 nmol per μ g of DNA (panel G and H) resulted in less stability.

Figure 8. In Vivo Gene Expression Mediated by Polyacridine Peptide DNA Polyplexes

The gene transfer efficiencies of naked DNA (●) or polyacridine peptide DNA polyplexes prepared with 0.2 (▲), 2 (○), or 4 (△) nmol of PEG-Cys-(Lys-(Acr))₅ per μg of DNA were determined following i.m. dosing and electroporation of 1 μg of pGL3 in the gastrocnemius muscle of ICR male mice (n=4). The luciferase expression was quantified at times ranging from 2–14 days by bioluminescence imaging (BLI) following an i.m. dose of luciferin. Open polyplexes prepared at 0.2 nmol (▲) of PEG-Cys-(Lys-(Acr))₅ per μg of DNA showed the lowest transfection efficiency, while polyplexes prepared at 2 nmol (○) of PEG-Cys-(Lys-(Acr))₅ per μg of DNA showed similar expression levels to that of naked DNA. Increasing the stoichiometry to 4 nmol (△) PEG-Cys-(Lys-(Acr))₅ per μg of DNA resulted in a more sustained expression. The results represent the mean and standard deviation of four doses.

Table 1

MS Analysis of Polyacridine PEG-Peptides and Mono and Bis-Acridine PEG

Sample	Mass (g/mol)	Mass Difference ^b
Mono-amine-PEG	5385	
Mono-acridine-PEG	5626	241 (0.8) ^c
Bis-amine-PEG	2000	
Bis-acridine-PEG	2813	813 (2.8) ^c
Cys-Trp-Lys ₃ ^a	691.4	
PEG-Cys-Trp-Lys ₃	6524	
PEG-Cys-Trp-(Lys(Acr)) ₃	7238	714 (2.5) ^c
Cys-Trp-Lys ₄ ^a	819.5	
PEG-Cys-Trp-Lys ₄	6623	
PEG-Cys-Trp-(Lys(Acr)) ₄	7533	910 (3.1) ^c
Cys-Trp-Lys ₅ ^a	948.5	
PEG-Cys-Trp-Lys ₅	6555	
PEG-Cys-Trp-(Lys(Acr)) ₅	7822	1267 (4.4) ^c

^a Determined by ESI-MS.^b Measured mean difference in MALDI-TOF mass due to the addition of acridine.^c Calculated number of acridine-6-amino-hexanoic acids based on 291 g/mol.