Synthesis and In Vitro Testing of New Potent Polyacridine−Melittin Gene Delivery Peptides

Nicholas J. Baumhover, Kevin Anderson, Christian A. Fernandez, and Kevin G. Rice*

Divisions of Medicinal and Natural Products Chemistry and Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242. Received July 14, 2009; Revised Manuscript Received October 28, 2009

The combination of a polyacridine peptide modified with a melittin fusogenic peptide results in a potent gene transfer agent. Polyacridine peptides of the general formula $(Acr\text{-}X)_n$-Cys were prepared by solid-phase peptide synthesis, where Acr is Lys modified on its ε-amine with acridine, X is Arg, Leu, or Lys and n is 2, 3, or 4 repeats. The Cys residue was modified by either a maleimide−melittin or a thiolpyridine-Cys-melittin fusogenic peptide resulting in reducible or non-reducible polyacridine−melittin peptides. Hemolysis assays established that polyacridine−melittin peptides retained their membrane lytic potency relative to melittin at pH 7.4 and 5. When combined with plasmid DNA, the membrane lytic potency of polyacridine−melittin peptides was neutralized. Gene transfer experiments in multiple cell lines established that polyacridine−melittin peptides mediate expression as efficiently as PEI. The expression was very dependent upon a disulfide bond linking polyacridine to melittin. The gene transfer was most efficient when X is Arg and n is 3 or 4 repeats. These studies establish polyacridine peptides as a novel DNA binding anchor peptide.

INTRODUCTION

An important element of many polyplex nonviral gene delivery systems is a releasable fusogenic peptide that can effect endosomal lysis (*1*). The endosomal escape of DNA is especially essential for receptor-targeted gene delivery systems to avoid polyplex degradation by the lysosome (*2*−*4*).

To improve the endosomal escape of DNA, several prior studies have incorporated fusogenic peptides, including GALA, KALA, HA2, JTS-1, and melittin, into a nonviral gene delivery system (*5*−*11*). Most applications in gene delivery have utilized melittin because of its relative ease of chemical synthesis and because its mechanism of membrane lysis has been extensively studied (*12*−*14*). Melittin has been covalently attached to polylysine, PEI, and cationic lipids and even self-polymerized by terminal disulfide bonds and demonstrated to increase in vitro gene transfer efficiency (*4, 10, 11, 15, 16*).

While DNA anchoring polymers such as PEI, polylysine, dendrimers, and chitosan are known to bind ionically to the phosphate backbone of plasmid DNA, this strategy leads to the formation of cationic polyplexes that favor in vitro gene transfer, but to a much lesser degree in vivo gene transfer (*17*). This is partly because positively charged polyplexes bind non-specifically to proteins and cells (*18*). Attempts at masking the charge with PEG still results in a residual positive charge as measured by zeta potential (*19, 20*). The non-specific binding of PEGylated cation polyplexes in vivo complicates strategies that attempt to combine a receptor ligand and releasable fusogenic peptide into a non-viral delivery system (*21, 22*). Consequently, it would be beneficial to reversibly bind fusogenic peptides, receptor ligand, and PEG to DNA while simultaneously controlling the charge of polyplexes. Likewise, it would be advantageous if the DNA binding anchor were sufficiently small in size to allow for its chemical manipulation and specific functionalization with PEG, targeting ligand, and fusogenic peptide (*22*).

Polyacridine peptides have been shown to reversibly bind with high affinity by intercalating into double-stranded DNA (*23*). An early study by Szoka demonstrated the synthesis of a divalent acridine neoglycopeptide for delivery of DNA to the cells expressing the asialoglycoprotein receptor (*24*). More recently, polyacridine-nuclear localization sequences (NLS) possessing up to three acridines were shown to improve in vitro gene transfer (*25, 26*).

However, to date, a polyacridine DNA binding peptide has not been substituted with a fusogenic peptide. The new strategy described herein demonstrates the synthesis of polyacridine−melittin as a DNA binding peptide and establishes its efficacy as a potent in vitro gene transfer agent. The results demonstrate that short polyacridine anchor peptides bind to plasmid DNA with sufficient affinity to deliver melittin in vitro and have the potential to control DNA polyplex charge to allow improved in vivo gene delivery.

MATERIALS AND METHODS

Unsubstituted Wang resin for peptide synthesis, 9-hydroxybenzotriazole, Fmoc-protected amino acids, *O*-(benzotriazole)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1,3-diisopropylcarbodiimide (DIC), Fmoc-Lysine-OH, and *N*-methyl-2-pyrrolidinone (NMP) were obtained from Advanced ChemTech (Lexington, KY). *N*,*N*-Dimethylformamide (DMF), trifluoroacetic acid (TFA), and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Diisopropylethylamine, piperdine, acetic anhydride, tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 9-chloroacridine, maleic anhydride, 2,2′-dithiodipyridine (DTDP), and thiazole orange were obtained from Sigma Chemical Co. (St. Louis, MO). Polyethylene amine (PEI 25 KDa) was purchased from Aldrich (Milwaukee, WI). D-Luciferin and luciferase from *Photinus pyralis* were obtained from Roche Applied Science (Indianapolis, IN). HepG2, CHO, and 3T3 cells were acquired from the American Type Culture Collection (Manassas, VA). Inactivated qualified fetal bovine serum (FBS) was from Life Technologies, Inc. (Carlsbad, CA). BCA reagent was purchased from Pierce (Rockford, IL). pGL3 control vector, a 5.3 kb luciferase plasmid containing a SV40 promoter and enhancer, was obtained from Promega (Madison,

* To whom correspondence should be addressed. E-mail kevin-rice@uiowa.edu. tel: 319-335-9903; fax: 319-335-8766.

10.1021/bc9003124
Published on Web 12/07/2009

WI). pGL3 was amplified in a DH5α strain of *Escherichia coli* and purified according to manufacturer's instructions.

Synthesis of 9-Phenoxyacridine and Fmoc Lysine (Acridine). 9-Phenoxyacridine was synthesized with modification from the methods of Tung et al. (*27*). Briefly, 12 g of phenol (127.5 mmol) and 0.72 g of sodium hydroxide (18 mmol) were heated to 100 °C. To the liquefied phenol, 2.8 g of 9-chloroacridine (13.105 mmol) was added and stirred vigorously for 1.5 h. The reaction was quenched by the addition of 100 mL of 2 M sodium hydroxide, then allowed to sit at RT overnight. A yellow precipitate was filtered, washed with water, and dried in vacuo (3.4822 g, 12.835 mmol, 97.9%, m.p. 123−124 °C, TLC 15:5:1:0.5 ethyl acetate/methanol/hexane/acetic acid, $R_f = 0.18$). ^{1}H NMR (DMSO-d_6) δ 8.23 (d, 2H), 8.04 (d, 2H), 7.88 (t, 2H), 7.59 (t, 2H), 7.32 (t, 2H), 7.08 (t, 1H), 6.88 (d 2H).

Fmoc-Lysine(Acridine)-OH was prepared by adding 2.18 g of Fmoc-Lys-OH (5.91 mmol) in liquid phenol (6.781 g, 73.01 mmol) to 9-phenoxyacridine (3 g, 11.06 mmol), then heated at 60 °C for 4 h under an argon atmosphere. Diethyl ether (80 mL) was then added while stirring vigorously until a yellow precipitate formed that was immediately recovered by filtration and washed repeatedly with diethyl ether. The product was allowed to dry overnight under vacuum (2.90 g, 5.32 mmol, 90%), m.p. 135−140 °C, TLC 1:1, 0.1 v/v % TFA/acetonitrile, $R_f = 0.75$). MS: $(M + H^+)^{1+} = 545.5$ *m/z*. ^{1}H NMR (DMSO-d_6) δ 6.7−7.8 (m, 17H), 3.6−3.95 (m, 2H), 3.25−3.53 (br, 3H), 2,06 (m, 1H), 0.8−1.5 (m, 6H).

Synthesis of Maleimide Glycine (Mal-Gly-OH). Glycine (5 g, 66.6 mmol) and maleic anhydride (6.6 g, 66.6 mmol) were suspended in 80 mL of acetic acid and allowed to react for 3 h at RT. The resulting white precipitate was collected by filtration, washed with cold water, and dried (10.95 g, 63.3 mmol, 95%, m.p. 187−189 °C, TLC 2:1:1:1 isopropyl alcohol/acetic acid/ethyl acetate/water, $R_f = 0.5$). The glycine maleic acid intermediate was characterized by proton NMR (300 MHz, DMSO-d_6): $\delta = 9.2$ (s, 1H), 6.397 (d, 1H, $j = 8.57$ Hz), 6.310 (d, 2H, $j = 12.86$ Hz), 2.0 (d, 2H, $j = 6.86$ Hz).

Glycine maleamic acid (5.2 g, 30.04 mmol) and 2.1 equiv of triethylamine (6.37 g, 63 mmol) were refluxed for 3 h in 500 mL toluene with removal of water with a Dean−Stark apparatus. Upon reaction completion, the toluene solution was decanted and dried. The resulting solid was acidified with 2 M HCl, extracted with ethyl acetate, dried with $MgSO_4$, and evaporated to yield Mal-Gly-OH (1.7 g, 11 mmol, 36.5%, m.p. 99−110 °C, TLC 2:1:1:1 isopropyl alcohol/acetic acid/ethyl acetate/water, $R_f = 0.72$). The product was characterized by proton NMR (300 MHz, DMSO-d_6): $\delta = 7.108$ (s, 2H), 4.105 (s, 2H) (*28*).

Synthesis and Characterization of Polyacridine−Melittin Analogues. Melittin analogues, polyacridine peptides, and control peptides were prepared by solid-phase peptide synthesis using standard Fmoc procedures with 9-hydroxybenzotriazole and HBTU double couplings on a 30 μmol scale on an Advanced ChemTech APEX 396 synthesizer. Mal-melittin was prepared by coupling Mal-Gly-OH to the N-terminus of sidechain protected full-length melittin on resin utilizing a 6-fold excess of Mal-Gly-OH, DIC, and HOBt, reacted for 4 h while stirring. The resin was filtered; washed with DMF, DCM, and methanol; then dried. Peptides were removed from resin and sidechain deprotected using a cleavage cocktail of TFA/triisoproylsilane/water (95:2.5:2.5 v/v/v) for 3 h followed by precipitation in cold ether. Precipitates were centrifuged for 10 min at 4000 rpm 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 semipreparative column (2 × 25 cm) eluted at 10 mL/min with 0.1 v/v % TFA with an acetonitrile gradient of 20−45 v/v % over 30 min while monitoring tryptophan (Abs 280 nm) or acridine (Abs 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 (tryptophan $\varepsilon_{280nm} = 5600$ M^{-1} cm^{-1}, thiolpyridine, and tryptophan $\varepsilon_{280nm} = 10\,915$ M^{-1} cm^{-1}, or acridine $\varepsilon_{409nm} = 9266$ M^{-1} cm^{-1}) to determine isolated yield. Purified peptides were characterized by LC-MS by injecting 2 nmol onto a Vydac C18 analytical column (0.47 × 25 cm) eluted at 0.7 mL/min with 0.1 v/v % TFA and an acetonitrile gradient of 10−55 v/v% over 30 min while acquiring ESI-MS in the positive ion mode.

Cys-melittin was reacted with 10 equiv of DTDP in 2-propanol/2 N acetic acid (10:3) for 8 h at RT to generate the thiolpyridine protected peptide. The reaction mixture was concentrated by rotary evaporation and applied to a Sephadex G-10 column eluted (2.5 × 50 cm) with 0.1 v/v % TFA while monitoring absorbance at 280 nm to remove excess DTDP. The peptide peak fractions were pooled, concentrated, lyophilized, and purified to homogeneity by RP-HPLC as described previously.

Poly(Acr-X)$_n$-SS-melittin and poly(Acr-X)$_n$-Mal-melittin peptides were synthesized on a 2 μmol scale based on melittin, using 1.7 equiv (3.4 μmol) of the reduced poly(Acr-X)$_n$-Cys, where X equals Arg, Lys, or Leu. Mal-melittin (2 μmol) was prepared in 8 mL of 10 mM ammonium acetate pH 5 and added to poly(Acr-X)$_n$-Cys and 2 mL of methanol to facilitate solubility. The reaction occurred over 12 h at RT as determined by analyzing aliquots by LC-MS. At completion, the reaction mixture was concentrated by rotary evaporation and lyophilized prior to purification of poly(Acr-X)$_n$-Mal-melittin by RP-HPLC eluted with 0.1 v/v % TFA and acetonitrile gradient (35−45% over 30 min) while detecting at 409 nm.

Alternatively, thiolpyridine-Cys-melittin (2 μmol) was prepared in 8 mL of 10 mM ammonium acetate pH 6 and added to 3.4 μmol poly(Acr-X)$_n$-Cys in 2 mL of methanol. After 24 h, poly(Acr-X)$_n$-SS-melittin was purified and characterized by LC-MS as described above.

Polyacridine−Melittin Hemolysis Assay. Whole blood was obtained from male ICR mice by heart puncture with heparinized 22G needles and collected in conical tubes containing 10 mL of 0.15 M PBS (pH 7.4) prewarmed to 37 °C. Erythrocytes were immediately separated from plasma by centrifugation at 2000 rpm for 2 min, washed three times with 10 mL of PBS, and then diluted to 1.5 × 10^8 cells/mL. Peptides were prepared at 15 μM and serially diluted to 10−0.01 μM, after which 100 μL was pipetted in triplicate into a MultiScreenHTS BV 96-well plate. Erythrocytes (50 μL, 7.5 × 10^6 cells) were added to the peptides and incubated at 37 °C for 1 h followed by filtration on a Multiscreen vacuum manifold (Millipore Corporation, Billerica, MA). The filtrate was measured for abs$_{410nm}$ on a Biotech plate reader (Biotech Instruments Incorporated., Winooski, VT) and compared to the 100% hemolysis caused by replacing PBS with water. Peptide DNA polyplexes (1.5 nmols and 7.5 μg pGL3) were also assayed for hemolysis as described above.

Formulation and Characterization of Polyacridine−Melittin Polyplexes. The relative binding affinity of peptides for DNA was determined by a fluorophore exclusion assay (*29*). pGL3 (200 μL of 4 μg/mL in 5 mM Hepes pH 7.5 containing 0.1 μM thiazole orange) was combined with 0, 0.05, 0.1, 0.25, 0.35, 0.5, 1, and 2 nmols of 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 DNA was subtracted from all values before data analysis. The data are presented as nmol of peptide per μg of DNA versus the percent fluorescence intensity plus/minus the standard deviation determined by three independent measurements.

Particle size and zeta potential were determined by preparing 2 mL of polyplex in 5 mM Hepes pH 7.5 at a DNA concentration of 30 μg/mL with 15 nmols/mL of peptide (0.5 nmol of peptide per μg of DNA). 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.

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 (*30*). Alternatively, naked DNA (100 μg pGL3 per mL) was prepared in 10 mM Tris, 1 mM EDTA pH 8, then diluted to 1 μg per mL in 40 mM Hepes 5 mM nickel chloride pH 6.7, and deposited on a fresh cleaved mica surface for 10 min followed by washing with deionized water. An Asylum atomic force microscope (AFM) MFP3D (Santa Barbara, CA) was operated in the AC-mode in order to image either naked DNA or DNA polyplexes using a silicon cantilever (Ultrasharp NSC15/AIBS, Mikro Masch).

Polyacridine−Melittin Polyplex Toxicity. The in vitro toxicity of polyacridine−melittin polyplexes was evaluated by MTT assay (*31*). CHO, HepG2, and 3T3 cells were plated on 6 × 35 mm wells at 5 × 10^5 cells/well and grown to 40−70% confluency. The media was replaced with 2 mL of fresh MEM supplemented with 2% FBS and the cells treated with 10 μg of DNA polyplex (0.5 nmol peptide/μg DNA) in HBM (HBM; 5 mM Hepes, 0.27 M mannitol, pH 7.5). After 6 h, the media was replaced with fresh culture media and grown an additional 18 h. Alternatively, cells were treated with DNA polyplexes for 24 h. The media was replaced with 2 mL of fresh media and 500 μL of 0.5% (w/v) 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in PBS solution and allowed to incubate at 37 °C for 1 h to let formazan crystals form. The media containing MTT was removed, and the crystals dissolved by the addition of 2 mL of dimethyl sulfoxide (DMSO) and 250 μL Sorenson's Glycine Buffer, then measured by absorbance 595 nm on a microplate reader. The percent viability was determined relative to untreated cells.

In Vitro Gene Transfer of Polyacridine−Melittin DNA Polyplexes. HepG2 cells (5 × 10^5) were plated on 6 × 35 mm wells and grown to approximately 50% confluency. Transfections were performed in MEM supplemented with either 2% or 10% FBS, sodium pyruvate (1 mM), and penicillin and streptomycin (100 U and 100 μg/mL). Polyplexes were prepared at a DNA concentration of 30 μg/mL and a stoichiometry of 0.5 nmol peptide per μg of DNA in HBM (HBM; 5 mM Hepes, 0.27 M mannitol, pH 7.5). Polyplexes (10 μg of DNA in 0.3 mL of HBM) were added dropwise to wells in triplicate and incubated 24 h. After 24 h, the cells were washed twice with 2 mL of ice-cold phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) and then treated with 0.5 mL of lysis buffer (25 mM Tris chloride, pH 7.8, 1 mM EDTA, 8 mM magnesium chloride, and 1% Triton X-100) for 10 min at 4 °C. Cell lysates were scraped, transferred to 1.5 mL microcentrifuge tubes, and centrifuged for 10 min at 13,000 *g* at 4 °C to pellet cell debris. Lysis buffer (300 μL) and sodium-ATP (4.3 μL of a 165 mM solution at pH 7, 4 °C) were combined in a test tube, mixed briefly, and immediately placed in the luminometer. Luciferase relative light units were measured by a Lumat LB 9501 (Berthold Systems, Germany) with 10 s integration after automatic injection of 100 μL of 0.5 mM D-luciferin. The relative light units were converted to fmol using a standard curve generated by adding a known amount of luciferase to 35 mm wells containing 50% confluent HepG2 cells. The resulting standard curve had an average slope of 2.6 × 10^4 RLU/fmol enzyme. Protein concentrations were measured by a BCA assay using bovine serum albumin as a standard (*32*). The amount of luciferase recovered in each sample was normalized to mg of protein and reported as the mean and standard deviation obtained from triplicate transfections. PEI pGL3 polyplexes were prepared by mixing 35 μg of DNA in 525 μL of HBM with 43.8 μg PEI in 525 μL of HBM while vortexing to create DNA complexes possessing a charge ratio (NH_4^+/PO_4^-) of 9:1. Cells were transfected with 10 μg PEI−DNA polyplexes as previously described. CHO and 3T3 (5 × 10^5) cells were plated on 6 × 35 mm wells and grown to ca. 50% confluency and then transfected as described above.

RESULTS

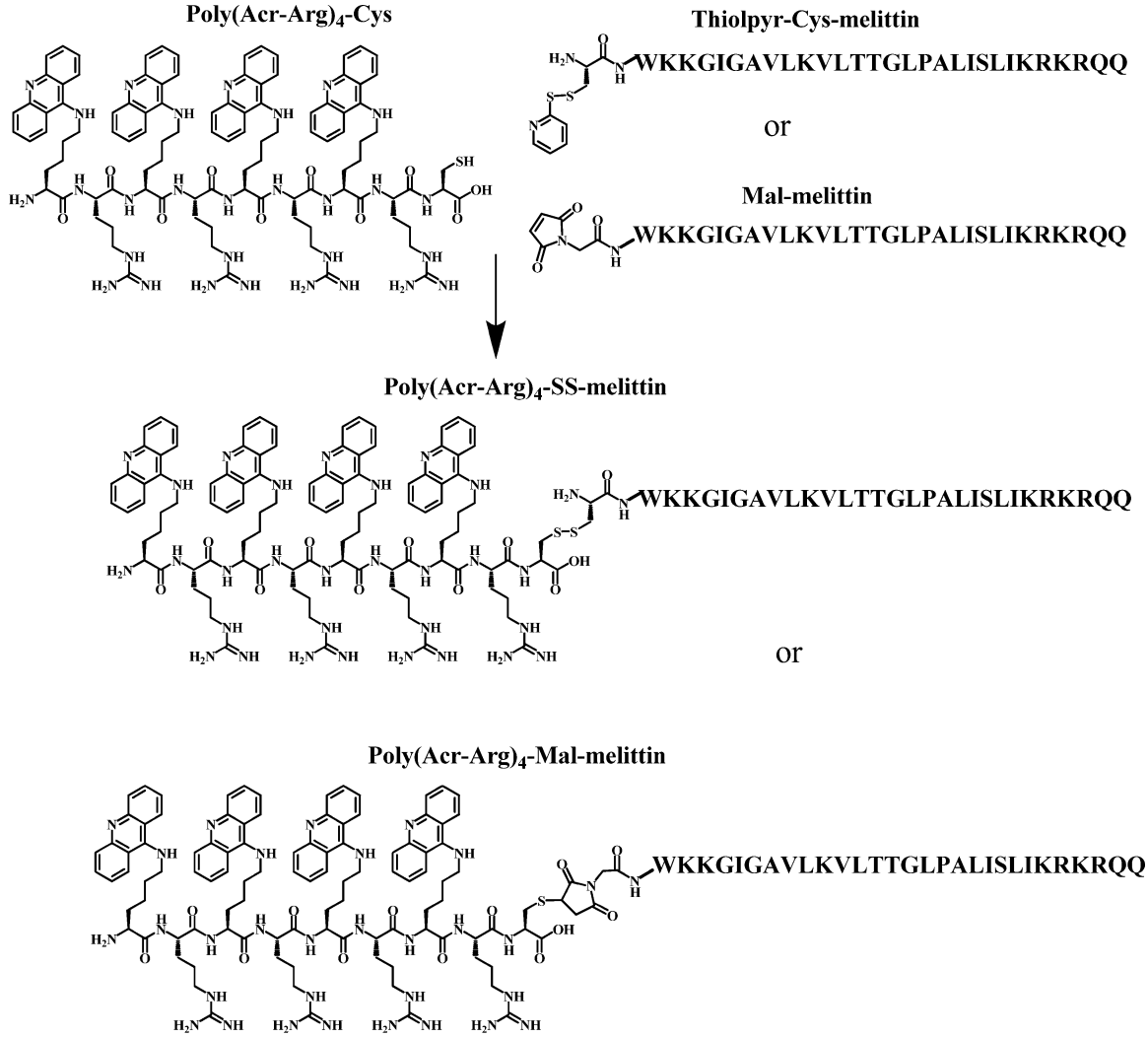
Synthetic Strategy for Polyacridine−Melittin. Naturally occurring melittin isolated from bee venom is a 26 amino acid peptide amide composed of two α-helices conjoined through an interrupting proline (*12*−*14*). Numerous studies have been conducted to investigate the mechanism and sequence specificity of melittin's membrane lytic activity.

Natural melittin contains a single aromatic Trp at residue 8 and does not contain a Cys residue. To improve the synthesis of polyacridine−melittin, we replaced Trp 8 with Leu, and reinstalled a single Trp near the N-terminus in Cys-Trp-Lys-Lys. The reactivity of the Cys residue was increased by flanking Lys residues. This also allowed selective chromatographic detection of full-length melittin at 280 nm. These modifications resulted in a Cys-melittin analogue, CWKKGIGAVLKVLTTGLPALISLIKRKRQQ, that SOPMA analysis (*33*) predicted to maintain α-helical character. A similar Mal-melittin analogue (Mal-GWKKGIGAVLKVLTTGLPALISLIKRKRQQ), possessing an N-terminal maleimide-glycine (Mal-G) in place of Cys, was also prepared.

Polyacridine and melittin peptides were synthesized using HBTU/HOBt with increased coupling efficiency compared to DIC. Early attempts to synthesize polyacridine peptides using DIC resulted in heterogeneous mixtures of truncated peptides with minimal recovery of full-length peptide. The identity of the spacing amino acid between Lys-acridine residue significantly influenced the synthetic yield. Bulky amino acid side chains and protecting groups such as Lys-Boc, Phe-Trt, Leu, Glu-OBut, and Arg-Pbf were well-tolerated, whereas Gly surprisingly was not. Polyacridine peptides possess a C-terminal Cys to accommodate bioconjugation to Cys-melittin or Mal-melittin. Polyacridine peptides possessing an alternating Lys-Acr (Acr) spaced by Arg, Lys, or Leu were designed to influence the charge character of the anchor peptide (Scheme 1 and Table 1). The lengths of polyacridine peptides were systematically varied to possess 2−4 Acr-Arg repeats to examine the influence of affinity. Polyacridine nona-peptides were routinely obtained in 30−35% purified yield with >95% purity.

Polyacridine−melittin peptides were designed to possess either a reducible dithiol or a nonreducible maleimide linkage (Scheme 1 and Table 1). The most expedient synthetic route proved to be cleavage of Cys-melittin from the resin, followed by modification with dithiol dipyridine to produce a thiolpyr-melittin. The RP-HPLC monitoring of the reaction of thiolpyr-melittin with poly(Acr-Arg)$_4$-Cys is illustrated in Figure 1A and B. The desired poly(Acr-Arg)$_4$-SS-melittin product is formed with complete consumption of thiolpyr-melittin, along with formation of a poly(Acr-Arg)$_4$-Cys$_2$ byproduct (Figure 1B). This

Scheme 1. Synthesis of Polyacridine−Melittin Peptides



Thiolpyr-melittin is reacted with the DNA binding anchor peptide poly(Acr-Arg)$_4$-Cys at pH 6 to yield poly(Acr-Arg)$_4$-SS-melittin as a reducible gene transfer polyacridine melittin peptide. Alternatively, Mal-melittin is reacted with poly(Acr-Arg)$_4$-Cys at pH 5 to yield a non-reducible poly(Acr-Arg)$_4$-Mal-melittin gene transfer peptide. In each case, Acr refers to a Lys substituted on its ε-amine with acridine. The Arg was substituted with Lys and Leu to produce DNA binding anchor peptides with different affinity. The poly(Acr-Arg)$_n$ was also varied from $n = 2-4$ repeats.

synthetic route resulted in poly(Acr-X)$_n$-SS-melittin in 35−50% purified yield at >95% purity as illustrated by LC-MS analysis of purified poly(Acr-Arg)$_4$-SS-melittin (Figure 1C). ESI-MS was used to establish structure based on the triple and quadruple charged positive ions corresponding to the calculated mass of the desired polyacridine−melittin peptide (Figure 1C, inset). The release of melittin from poly(Acr-Arg)$_4$-SS-melittin was established by reaction with TCEP to regenerate poly(Acr-Arg)$_4$-Cys and Cys-melittin peptide peaks (Figure 1D).

Poly(Acr-X)$_4$-Mal-melittin peptides, where X is Arg, Lys, or Leu, were synthesized to compare their gene transfer properties with poly(Acr-Arg)$_4$-SS-melittin (Scheme 1 and Table 1). The synthetic strategy utilized Gly-maleimide that was coupled to the N-terminus of the fully protected melittin peptide on resin (Scheme 1). Upon workup, this yielded Mal-melittin that was then directly conjugated to poly(Acr-X)$_n$-Cys peptides. The conjugation of Mal-melittin with poly(Acr-Arg)$_4$-Cys was monitored by RP-HPLC resulting in the complete consumption of Mal-melittin and the formation of poly(Acr-Arg)$_4$-Mal-melittin. Purification of poly(Acr-Arg)$_4$-Mal-melittin was achieved with a final yield of 35% and >95% purity. Reducible and nonreducible polyacridine−melittin peptides with Acr-Arg repeat of 2 and 3 were synthesized with similar yield and purity, and characterized by LC-MS (Table 1).

Physical Properties of Polyacridine−Melittin DNA Polyplexes. The binding of polyacridine−melittin peptides to pGL3 was investigated using a thiazole orange exclusion assay (*29*). Polyacridine−melittin peptides were found to compete for intercalation and displace thiazole orange resulting in a decrease in fluorescence intensity. When titrated at increasing concentration, the fluorescence decreased until an asymptote was reached at an equivalence point (Figure 2A). Analysis of the DNA binding affinity of poly(Acr-Arg)$_4$-Cys established an equivalence point at approximately 0.4 nmol peptide per μg of DNA. By comparison, Cys-melittin demonstrated weaker binding and was not able to fully exclude thiazole orange, only approaching an equivalence point at approximately 0.5 nmol of peptide per μg of DNA (Figure 2A). Alternatively, poly(Acr-Arg)$_4$-SS-melittin demonstrated higher affinity resulting in an equivalence point at approximately 0.25 nmol of peptide per μg of DNA. This affinity is comparable to that afforded by a Cys-Trp-Lys$_{18}$ peptide using the same assay (*29*).

Comparison of poly(Acr-Arg)$_{2-4}$-SS-melittin demonstrated nearly equivalent affinity for Acr-Arg repeats of 3 and 4, but

Table 1. Sequence and Hemolytic Potency of Peptide Conjugates

		mass[a] (calc/obs)	HL_{50} (μM)[b] pH 7.4: 5.0
Polyacridine Anchor Peptides (Acr) = Lys-ε-acridine, Ac = acylated ε-amine)			
poly(Acr-Arg)$_2$-Cys		1044.3/1044.2	N.D.[c]
poly(Acr-Arg)$_3$-Cys		1505.8/1505.6	N.D.
poly(Acr-Arg)$_4$-Cys		1967.4/1967.0	>10/>10
poly(Acr-Lys)$_4$-Cys		1855.3/1855.0	N.D.
poly(Acr-Leu)$_4$-Cys		1795.3/1795.1	N.D.
Trp-(Lys(Ac)-Arg)$_4$-Cys		1612.9/1612.7	N.D.
Melittin Analogues (thiolpyr = thiol pyridine, Mal = maleimide)			
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	2847.5/2847.4	0.9/2.3
Cys-melittin	CWKKGIGAVLKVLTTGLPALISLIKRKRQQ	3320.1/3320.4	1.0/0.7
Dimeric-melittin	(CWKKGIGAVLKVLTTGLPALISLIKRKRQQ)$_2$	6638.2/6637.6	1.0/1.2
Thiolpyr-melittin	C(tp)WKKGIGAVLKVLTTGLPALISLIKRKRQQ	3429.2/3428.4	N.D.
Mal-melittin	(m)GWKKGIGAVLKVLTTGLPALISLIKRKRQQ	3354.0/3454.0	3.84/4.8
Polyacridine−Melittin (SS = reducible, Mal = nonreducible)			
poly(Acr-Arg)$_2$-SS-melittin	(Acr-Arg)$_2$-C−CWKKGIGAVLKVLTTGLPALISLIKRKRQQ	4359.5/4362.3	2.5/3.9
poly(Acr-Arg)$_3$-SS-melittin	(Acr-Arg)$_3$-C−CWKKGIGAVLKVLTTGLPALISLIKRKRQQ	4823.9/4823.2	1.4/2.2
poly(Acr-Arg)$_4$-SS-melittin	(Acr-Arg)$_4$-C−CWKKGIGAVLKVLTTGLPALISLIKRKRQQ	5285.5/5285.2	1.7/1.9
poly(Acr-Arg)$_4$-Mal-melttin	(Acr-Arg)$_4$-C(m)GWKKGIGAVLKVLTTGLPALISLIKRKRQQ	5321.4/5320.8	2.1/1.7
poly(Lys(Ac)-Arg)$_4$-SS-melttin	W(Lys(Ac)-Arg)$_4$-C−CWKKGIGAVLKVLTTGLPALISLIKRKRQQ	4931.0/4930.4	5.6/7.7
poly(Acr-Lys)$_4$-SS-melittin	(Acr-Lys)$_4$-C−CWK KG IGAVLKVLTTGLPALISLIKRKRQQ	5173.4/5173.2	1.0/2.8
poly(Acr-Lys)$_4$-Mal-melittin	(Acr-Lys)$_4$-C(m)GWKKGIGAVLKVLTTGLPALISLIKRKRQQ	5209.4/5208.8	1.5/1.9
poly(Acr-Leu)$_4$-SS-melittin	(Acr-Leu)$_4$-C−CWK KG IGAVLKVLTTGLPALISLIKRKRQQ	5113.4/5112.8	2.2/1.6
poly(Acr-Leu)$_4$-Mal-melittin	(Acr-Leu)$_4$-C(m)GWKKGIGAVLKVLTTGLPALISLIKRKRQQ	5149.3/5149.2	2.4/1.2

[a] Calculated and observed mass as determined by ESI LC-MS. [b] RBC hemolysis at pH 7.4 and 5.0. [c] N.D. = not determined.

less affinity afforded for a repeat of 2 (Figure 2B). Direct comparison of the DNA binding affinity of poly(Acr-Arg)$_4$-SS-melittin with poly(Acr-Lys)$_4$-SS-melittin and poly(Acr-Leu)$_4$-SS-melittin established that there was a negligible difference, with each producing fully complexed polyplexes at 0.3 nmol per μg of pGL3 (Figure 2C).

The particle size and zeta potential of polyacridine−melittin polyplexes were determined by QELS analysis (Table 2). Poly(Acr-Arg)$_4$-SS-melittin and poly(Acr-Arg)$_4$-Mal-melittin produced polyplexes of 79−84 nm average diameter, respectively. Comparison of the size of polyplexes prepared with poly(Acr-Arg)$_3$-SS-melittin (126 nm) and poly(Acr-Arg)$_2$-SS-melittin (737 nm) established that shorter repeats produced larger particle size (Table 2). This result is consistent with earlier findings that indicated that shorter, low-affinity polylysine peptides (<13 residues) produced larger polyplexes, whereas polylysine of 18 repeats or longer produced polyplexes of approximately 80 nm in diameter.

Substitution of the spacing Arg for Lys or Leu in Acr-Arg repeats resulted in a minimal change in particle size (Table 2). This supports the hypothesis that polyintercalation, and not ionic binding, is primarily responsible for the binding affinity to DNA. To test this hypothesis, an analogue was prepared that replaced the acridine modified Lys with an acetyl on the ε-amine of lysine resulting in poly(Lys(Ac)-Arg)$_4$-SS-melittin. This analogue produced polyplexes of average diameter of 177 nm, suggesting a significantly lower affinity (Table 2).

To further validate the change in particle size determined by QELS, polyplexes were analyzed by atomic force microscopy (AFM) (*30*). Analysis of pGL3 by AFM demonstrated an open structure occupying 0.5−1 μm with no single morphology (Figure 3A). Polyplexes prepared with poly(Acr-Arg)$_2$-SS-melittin appeared to form clusters composed of several individual particles that account for the larger size determined by QELS (Figure 3B). In contrast, the AFM images for polyplexes prepared with poly(Acr-Arg)$_4$-SS-melttin (Figure 3C), poly(Acr-Leu)$_4$-SS-melittin (Figure 3D), and poly(Acr-Lys)$_4$-SS-melittin (Figure 3E) all appeared to be less aggregated resulting in overall smaller particle size. It is interesting to note that the zeta potentials of polyacridine−melittin polyplexes containing a spacing Arg, Lys, or Leu were approximately +25 mV (Table 2). This result indicated that the charge on melittin strongly influenced the overall charge of polyplexes prepared with different anchoring peptides.

Biological Activity of Polyacridine−Melittin DNA Polyplexes. The membrane lytic potency of polyacridine−melittin peptides were investigated using a RBC hemolysis assay (*9*). As anticipated, poly(Acr-X)$_4$-Cys anchor peptides alone were inactive in membrane lysis. Alternatively, modification of the N-terminus of natural melittin with Cys-Trp-Lys-Lys and substitution of Leu for Trp 8 to generate Cys-melittin, resulted in retention of RBC hemolytic (HL_{50}) potency at pH 7.4 relative to natural melittin (Figure 4A). Likewise, the hemolytic potency of poly(Acr-Arg)$_4$-SS-melittin was comparable to Cys-melittin, indicating the attachment of the polyacridine anchor had a negligible influence on the membrane lytic activity of Cys-melittin. The results for polyacridine−melittin peptides possessing either a reducible or non-reducible linkage are summarized in Table 1, establishing their nearly equivalent hemolytic potency at pH 7.4.

The hemolytic potency of polyacridine melittin peptides was also determined at pH 5 to simulate the pH of the endosome. In contrast to an earlier finding that C and N-terminal modification of natural melittin with Cys-(Lys)$_4$ resulted in the complete loss of hemolytic activity at pH 5 (*9*), Cys-melittin possessed an improved pH 5 RBC lytic potency relative to that of natural melittin (Figure 4B). Consequently, poly(Acr-Arg)$_4$-SS-melittin and poly(Acr-Arg)$_4$-Mal-melittin both possess potent hemolytic activity at pH 7.4 and 5 (Table 1). When combined with pGL3, Cys-melittin and Mal-melittin maintained their hemolytic activity at pH 7.4, demonstrating their weak association with DNA (Figure 4C). In contrast, the hemolytic activity of poly(Acr-Arg)$_4$-SS-melittin DNA polyplexes was completely neutralized due to the strong binding of the polyacridine peptide with DNA (Figure 4C). Reduction of the disulfide bond would decrease the binding affinity to DNA and thereby release hemolytic Cys-melittin.

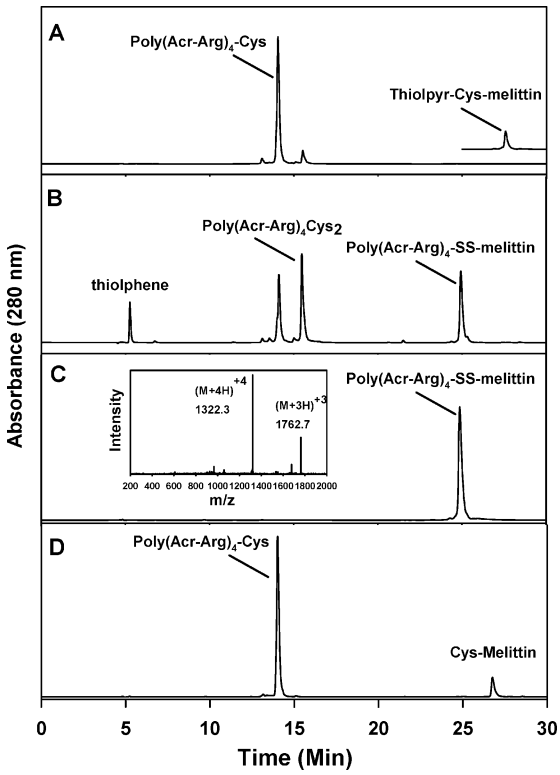


Figure 1. RP-HPLC analysis of polyacridine-melittin peptide synthesis. The reaction monitoring of poly(Acr-Arg)$_4$-Cys with thiolpyr-Cys-melittin at time 0 at 1.7:1 mol ratio is illustrated in panel A. The RP-HPLC chromatograms were produced by injecting 2 nmol then eluting with 0.1% TFA (v/v) and an acetonitrile gradient of 10−55% over 30 min while monitoring absorbance at 280 nm. The reaction monitoring at 12 h established the complete consumption of thiolpyr-Cys-melittin with formation of the product poly(Acr-Arg)$_4$-SS-melittin and by-products of dimeric poly(Acr-Arg)$_4$-Cys$_2$ and thiolphene (panel B). LC-ESI-MS of purified poly(Acr-Arg)$_4$-SS-melittin produced ions of 1762.7 *m*/*z* and 1322.3 *m*/*z*, respectively (inset), corresponding to a mass of 5285.2 amu (panel C). Reduction of poly(Acr-Arg)$_4$-SS-melittin with TCEP resulted in formation of equimolar amounts of poly(Acr-Arg)$_4$-Cys and Cys-melittin (panel D).

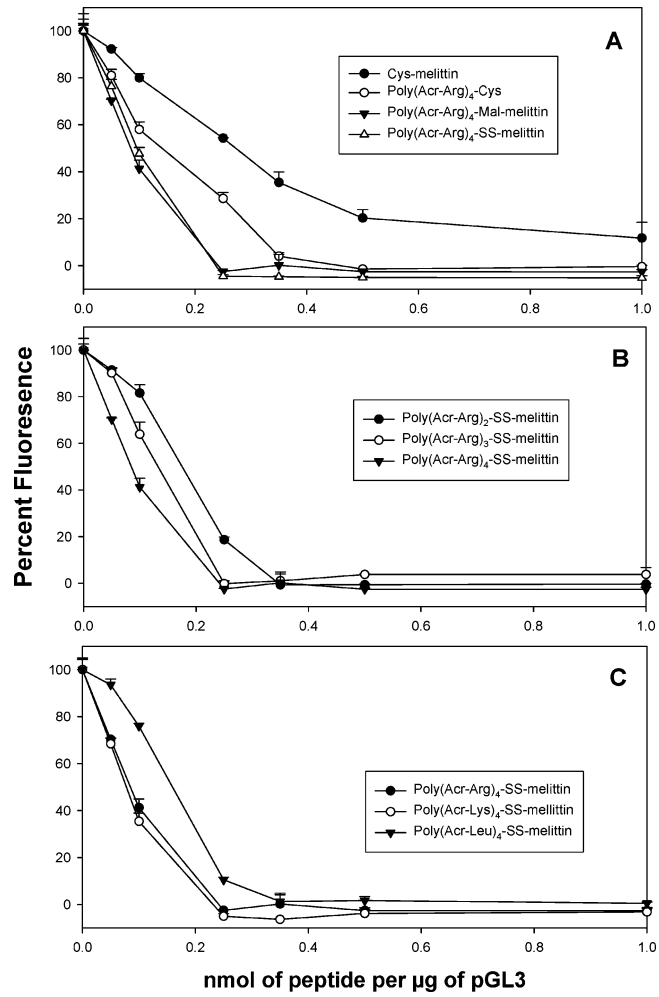


Figure 2. Relative binding affinity of polyacridine-melittin peptides to DNA. The concentration-dependent displacement of the thiazole orange from DNA by polyacridine−melittin peptides was used to establish relative affinity. Cys-melittin bound weakly to DNA resulting in an asymptote in the fluorescence intensity at approximately 0.5 nmol of peptide per μg of DNA, compared to 0.35 for (Acr-Arg)$_4$-Cys and 0.25 for poly(Acr-Arg)$_4$-SS-melittin (panel A). Comparison of poly(Acr-Arg)$_{2-4}$-SS-melittin established that poly(Acr-Arg)$_2$-SS-melittin was the weakest binding resulting in an asymptote at 0.35 nmols of peptide per μg of DNA, whereas poly(Acr-Arg)$_{3\text{and}4}$-SS-melittin were nearly equivalent in displacing thiazole orange (panel B). An equal DNA binding affinity was demonstrated for poly(Acr-Arg)$_4$-SS-melittin, poly(Acr-Leu)$_4$-SS-melittin, and poly(Acr-Lys)$_4$-SS-melittin, each resulting in polyplex formation at 0.25 nmol of peptide per μg of DNA (panel C).

To establish a relationship among peptide anchor length, reducibility, and peptide DNA stoichiometry, CHO cells were transfected with polyplexes formed with poly(Acr-Arg)$_2$-SS-melittin, poly(Acr-Arg)$_3$-SS-melittin, poly(Acr-Arg)$_4$-SS-melittin, and poly(Acr-Arg)$_4$-Mal-melittin while varying the peptide to DNA ratio from 0.1, 0.3, 0.5, and 0.75 nmols of peptide per μg of DNA (Figure 5). Luciferase reporter gene expression for polyplexes formed with 0.1 nmol of peptide per μg DNA resulted in expression comparable to a WK$_{18}$/DNA control polyplex. Increasing the stoichiometry to 0.35 nmols of peptide per μg of DNA or higher increased the gene expression approximately 100-fold relative to 0.1 nmol of peptide per μg of DNA. Within this series, poly(Acr-Arg)$_4$-SS-melittin produced the highest expression at a stoichiometry of 0.3 and 0.5. The expression mediated by poly(Acr-Arg)$_4$-SS-melittin was approximately 1000-fold greater than that of poly(Acr-Arg)$_4$-Mal-melittin, which differed only by the reducibility of the linkage between the anchor and melittin (Figure 5).

A broader panel of polyacridine−melittin peptides were combined with pGL3 at a fixed stoichiometry of 0.5 nmol per μg of pGL3 and used to mediate gene expression in HepG2, 3T3, and CHO cells. As illustrated in Figure 6, polyacridine−melittin peptides of the poly(Acr-Arg)$_{2-4}$-SS-melittin series produced the highest gene transfection in all three cell lines, with levels similar to that of PEI (9:1 N:P) when performed with either 2% or 10% FBS. By comparison, poly(Acr-Lys)$_4$-SS-melittin and poly(Acr-Leu)$_4$-SS-melittin were approximately 10−100-fold less active in gene transfer than poly(Acr-Arg)$_4$-SS-melittin in all three cell lines. Reducible poly(Acr-Arg)$_{2-4}$-SS-melittin peptides were 100−1000-fold more active in gene transfer compared to poly(Acr-Arg)$_4$-Mal-melittin in all three cell lines. The importance of the reductive release of melittin was also determined for poly(Acr-Lys)$_4$-SS-melittin and poly-(Acr-Leu)$_4$-SS-melittin in all three cell lines.

To establish the contribution of poly-acridine to DNA binding affinity and gene transfer, the ε-amine of lysine was modified with an acetyl group to prepare poly(Lys(Ac)-Arg)$_4$-SS-melittin. Gene transfer mediated by poly(Lys(Ac)-Arg)$_4$-SS-melittin was approximately 100−1000-fold less relative to poly(Acr-Arg)$_4$-SS-melittin in all three cell lines (Figure 6). To further establish the importance of the anchor peptides, Cys-melittin and dimeric-melittin were used to mediate gene transfer (Figure 6). Both mediated gene transfer that was 10−100-fold lower than poly(Acr-Arg)$_{2-4}$-SS-melittin, demonstrating the importance of the polyacridine anchor in this gene transfer system. Alterna-

Table 2. Physical Properties and Toxicity of DNA Polyplexes

peptide	particle size (nm)[a]	zeta potential (+mV)[b]	MTT assay[c] CHO	HepG2	3T3
poly(Acr-Arg)$_2$-SS-melittin	737 ± 46.8	25 ± 2.2	99 ± 7	92 ± 7	85 ± 2
poly(Acr-Arg)$_3$-SS-melittin	126 ± 4.5	29 ± 4.5	103 ± 7	97 ± 13	91 ± 2
poly(Acr-Arg)$_4$-SS-melittin	84 ± 2.0	29 ± 2.6	70 ± 5	67 ± 9	58 ± 4
poly(Acr-Arg)$_4$-Mal-melittin	79 ± 1.3	28 ± 2.5	69 ± 5	88 ± 13	82 ± 1
poly(Lys(Ac)-Arg)$_4$-SS-melittin	177 ± 6.2	20 ± 2.9	55 ± 4	64 ± 9	33 ± 4
poly(Acr-Lys)$_4$-SS-melittin	89 ± 3.5	23 ± 1.6	76 ± 5	66 ± 5	72 ± 6
poly(Acr-Lys)$_4$-Mal-melittin	86 ± 2.4	26 ± 2.0	87 ± 8	79 ± 6	82 ± 2
poly(Acr-Leu)$_4$-SS-melittin	119 ± 1.4	20 ± 2.9	46 ± 4	49 ± 4	50 ± 4
poly(Acr-Leu)$_4$-Mal-melittin	121 ± 2.5	22 ± 2.1	90 ± 3	117 ± 5	74 ± 3

[a] Mean particle size of peptide-DNA polyplex (0.5 nmol of peptide per μg of DNA) as determined by QELS. [b] Mean zeta potential determined in 5 mM Hepes, pH 7.4. [c] Percent viability of peptide-DNA polyplexes determined by MTT assay in CHO, HepG2, and 3T3 cells at 10 μg DNA dose (0.5 nmol of peptide per μg DNA) applied to 5×10^5 cells for 6 h.

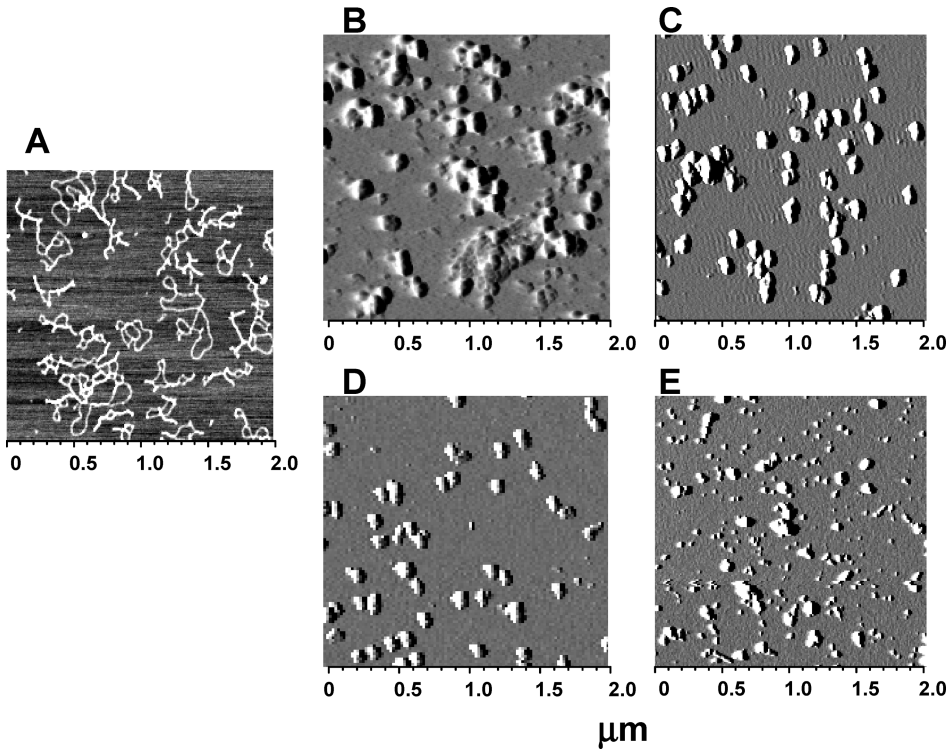


Figure 3. Atomic force microscopy of polyacridine−melittin polyplexes. The size and shape of pGL3 (A) or polyacridine−melittin DNA polyplexes (B−E) were determined by atomic force microscope. Poly(Acr-Arg)$_2$-SS-melittin (B), poly(Acr-Leu)$_4$-SS-melittin (C), poly(Acr-Arg)$_4$-SS-melittin (D), and poly(Acr-Lys)$_4$-Mal-melittin (E) pGL3 polyplexes are compared. The results establish that the weaker DNA binding of poly(Acr-Arg)$_2$-SS-melittin results in aggregated polyplexes.

tively, poly(Acr-Arg)$_n$Cys peptides produced negligible gene transfer on their own, establishing the importance of both the anchor peptide and melittin.

The toxicity of polyacridine DNA polyplexes was assessed by MTT assay on CHO, HepG2, and 3T3 cells (Table 2). Poly(Acr-Arg)$_{2-3}$-SS-melittin DNA polyplexes display very little toxicity in all three cell lines. However, poly(Acr-Arg)$_4$-SS-melittin and poly(Acr-Arg)$_4$-Mal-melittin polyplexes resulted in approximately 70% cell viability whereas control peptide poly(Lys(Ac)-Arg)$_4$-SS-melittin polyplexes were more toxic, resulting in 55% viability. Treatment of CHO, 3T3, and HepG2 cells with 10 μg of poly(Acr-Lys)$_4$-SS-melittin and poly(Acr-Lys)$_4$-Mal-melittin polyplexes for either 6 or 24 h resulted in the same degree of toxicity, whereas lowering the DNA polyplex dose to either 1 or 5 μg resulted in 100% viability. Despite its lower gene transfer efficiency, poly(Acr-Leu)$_4$-Mal-melittin polyplexes were less toxic compared to poly(Acr-Leu)$_4$-SS-melittin polyplexes, which produced approximately 50% viability (Table 2).

DISCUSSION

The endosome has been proposed as one of the major barriers that limits the gene transfer efficiency of nonviral gene delivery systems (*34*). To increase the endosomal escape of plasmid DNA, many prior studies have incorporated fusogenic peptides into experimental delivery systems (*4, 8, 16, 35−40*). Most often, a fusogenic peptide is linked to a PEI or polylysine to allow for multivalent reversible ionic binding with the phosphate backbone of DNA (*4, 10, 11*). While this approach often leads to significant increases in gene transfer efficiency in vitro, ionic interactions are relatively weak leading to premature dissociation of the carrier in vivo and degradation of the DNA (*41*). To overcome weak binding, higher molecular weight polycations are used. These can be prepared with reducible linkages that revert to low molecular weight, lower affinity polycations and

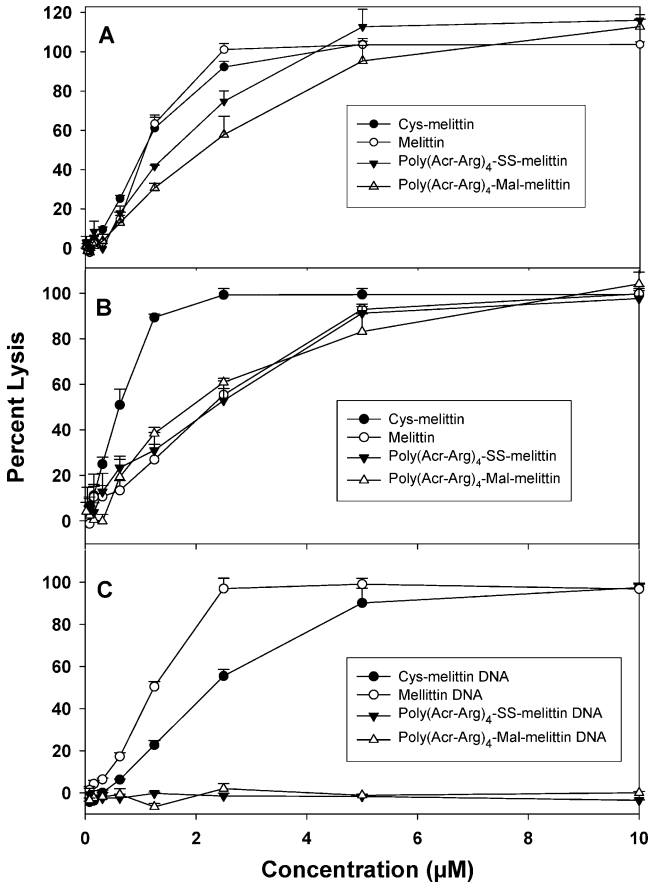


Figure 4. Membrane lytic potency of polyacridine−melittin peptides. The hemolytic activity of melittin and polyacridine−melittin peptides were compared at pH 7.4 and 5 using a RBC hemolysis assay. The results establish Cys-melittin is equally potent as natural melittin at pH 7.4 (panel A), but more potent at pH 5 (panel B). In comparison, poly(Acr-Arg)$_4$-SS-melittin retains nearly full hemolytic potency at pH 7.4 (panel A) but is less potent at pH 5. However, when combined with pGL3, the pH 7.4 hemolytic activity of both Cys-melittin and melittin is nearly unchanged, whereas poly(Acr-Arg)$_4$-SS-melittin is non hemolytic (panel C). These results establish the masking of poly(Acr-Arg)$_4$-SS-melittin membrane lytic activity while bound to DNA.

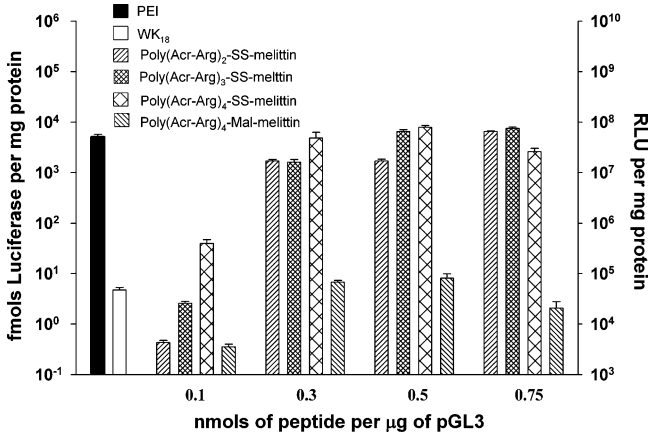


Figure 5. Comparative in vitro gene transfer potency of polyacridine−melittin polyplexes. CHO cells were transfected with increasing amounts of peptide (0.1, 0.35, 0.5, and 0.75 nmol peptide per microgram DNA) combined with 10 μg pGL3. The luciferase expression determined at 24 h represents the mean and standard deviation for three independent transfections.

trigger the release of DNA inside the cell (*21, 22, 42*−*45*). However, despite numerous attempts, i.v. dosed polycationic

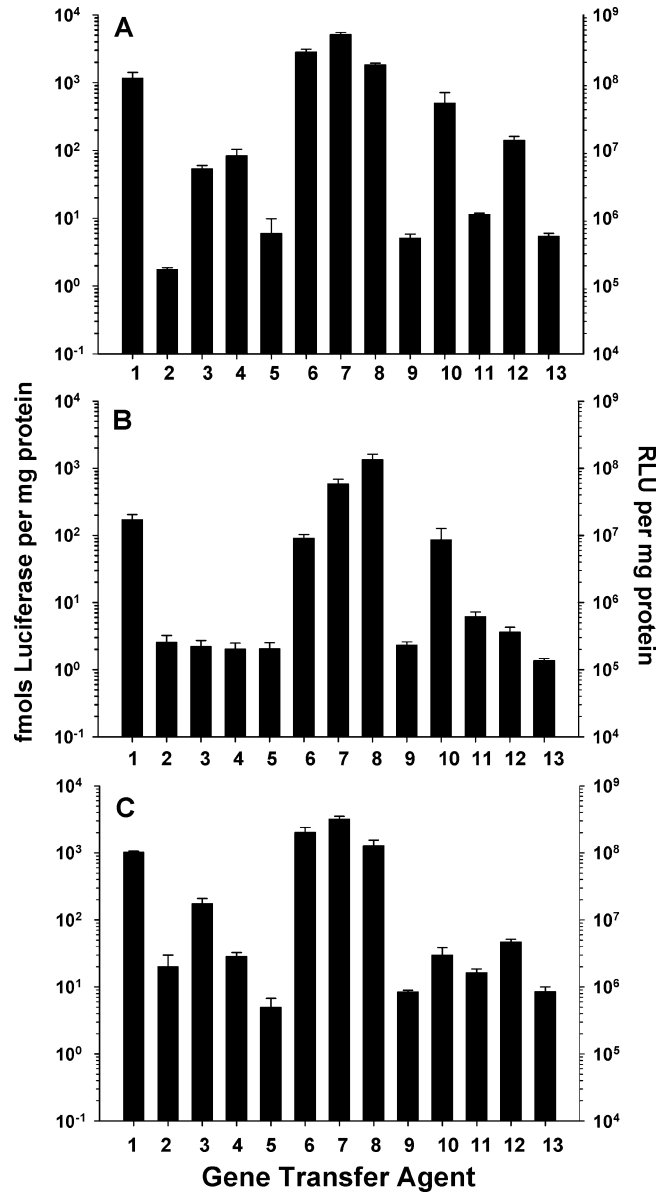


Figure 6. Cell-dependent in vitro gene transfer potency of polyacridine−melittin polyplexes. The relative gene transfer efficiency of DNA polyplexes was determined in CHO (A), 3T3 (B), and HepG2 (C) cells. Transfections were performed with 10 μg of pGL3 polyplex prepared with 0.5 nmol of peptide per μg of DNA. The gene transfer efficiency mediated by polyacridine−melittin peptides was compared to PEI (N/P 9:1) DNA polyplexes and WK$_{18}$ DNA polyplexes. The bars indicate transfection pGL3 combined with (1) PEI, (2)WK$_{18}$, (3) dimeric-melittin, (4) Cys-melittin, (5) poly(Lys(Ac)-Arg)$_4$-SS-melittin, (6) poly(Acr-Arg)$_2$-SS-melittin, (7) poly(Acr-Arg)$_3$-SS-melittin, (8) poly-(Acr-Arg)$_4$-SS-melittin, (9) poly(Acr-Arg)$_4$-Mal-melittin, (10) poly(Acr-Lys)$_4$-SS-melittin, (11) poly(Acr-Lys)$_4$-Mal-melittin, (12) poly(Acr-Leu)$_4$-SS-melittin, and (13) poly(Acr-Leu)$_4$-Mal-melittin. The luciferase expression was determined at 24 h. The results represent the mean and standard deviation for three independent transfections.

DNA polyplexes are unable to produce therapeutic levels of gene expression (*22*).

We have therefore explored an alternative method of binding fusogenic peptides to plasmid DNA by reversible intercalation. Preliminary studies suggested that a single acridine binds to DNA through intercalation but with relatively weak affinity. Consequently, we sought to increase the binding affinity by preparing polyacridines. Considering the pioneering studies of Szoka who developed a synthetic scheme to prepare a diacridine glycopeptide (*24*), Vierling with a single acridine modified nuclear localizing peptide (*26*), and Nielsen who prepared and

tested di and triacridine nuclear localization peptides (*25*), we sought a simplified approach that would allow us to extend the valency of polyacridine to four or more acridine units and control the charge of polyacridine DNA polyplexes. We therefore adopted a strategy reported by Ueyama et al. (*23*), who demonstrated that polyacridine peptides could be prepared from Fmoc-Lys(Acr) using solid-phase synthesis. Several early attempts at synthesis established that polyacridine peptides could be prepared in good yield (>30%) provided that coupling was conducted with HBTU and that a spacing amino acid was used that had a bulky side chain or protecting group. Using Gly as a spacing amino acid resulted in poor yields.

The synthetic design of polyacridine peptides took into account the relative DNA binding affinity by varying polyacridine repeat, the influence of the spacing amino acid on gene transfer and linkage between polyacridine and a melittin fusogenic peptide. As indicated in Figures 5 and 6, there is a strong dependency on the nature of the linkage between polyacridine and melittin in relationship to the gene transfer efficiency. A reducible disulfide bond provided a means for the triggered release of melittin either at or inside the cell. The reduction of the disulfide bond greatly diminishes the affinity of melittin for DNA, releasing it and unmasking its membrane lytic activity (Figure 2C). The released melittin apparently enhances gene transfer by lysis of endosomal membranes that then allows the DNA to more efficiently reach the cytosol and the nucleus, resulting in gene expression that equals or surpasses that of PEI in multiple cell lines (Figure 6). In support of this mechanism, polyacridine linked to melittin by a non-reducible maleimide is 1000-fold less active in gene transfer, despite its equivalent membrane lysis potency against RBCs (Table 1). These results agree with previously observed improved gene transfer mediated by a reductively triggered release of a fusogenic peptide from a gene delivery system (*10, 46*).

The length of the polyacridine repeat appears to be less important for in vitro gene transfer, since repeats of 2, 3, and 4 poly(Acr-Arg)-SS-melittin all produced nearly equivalent gene transfer in three cell lines (Figure 6). However, it is clear that poly(Acr-Arg)$_2$-SS-melittin has lower affinity for DNA as indicated by its ability to displace an intercalator dye (Figure 2B). The lower affinity results in larger DNA polyplexes as determined by QELS (Table 2) and AFM (Figure 3B). However, larger polyplexes also sediment more efficiently, facilitating in vitro gene transfer.

The spacing amino acid of Arg, Lys, or Leu within a poly(Acr-X)$_4$-SS-melittin only slightly influences their affinity for binding DNA (Figure 2C). Each of the resulting polyplexes possessed comparable size, charge, and shape as determined by QELS, zeta potential, and AFM (Table 2, Figure 3). However, the gene transfer efficiency was influenced significantly by the identity of the spacing amino acid in the order Arg > Lys > Leu (Figure 6). In each case, and in each cell line, the reducible polyacridine−melittin peptide mediated greater expression than the nonreducible analogue. The mechanism by which the spacing amino acid influences gene transfer efficiency is unclear. We speculate that the polyacridine and spacing amino acid collaborate to provide DNA binding affinity. It is likely that, following disulfide bond reduction to release the melittin, the anchoring peptide binds to DNA with less affinity, and the charge character of the polyplex changes significantly.

Nonetheless, it is clear that polyacridine is necessary to mediate significant gene transfer. This is further established with a control peptide, poly(Lys(Ac)-Arg)$_4$-SS-melittin, that was fully active in membrane lysis but proved to be approximately 1000-fold less active than poly(Acr-Arg)$_4$-melittin in mediating gene transfer (Figure 6).

The lytic potency of melittin on cells in culture has been well documented (*12, 14*). However, this toxicity can be overcome by polymerization of melittin through disulfide bonds resulting in its neutralization due to high-affinity binding to DNA (*9*). It is apparent that even dimeric-melittin can bind to DNA and mediate moderate gene transfer (Figure 6). The MTT assay did establish that polyacridine−melittin peptides are moderately toxic (approx 50−70% viable) when added to DNA at 0.5 nmol of peptide per microgram of DNA or higher (Table 2). However, this toxicity is the result of saturation of the DNA with polyacridine peptide resulting in unbound peptide that can lyse cells. Lower stoichiometries of 0.3 nmol of peptide per microgram of DNA or lower DNA doses of 5 and 1 μg result in negligible toxicity on all three cell types.

In conclusion, we have described a new polyacridine DNA binding anchor that, when linked with melittin through disulfide bond, produces potent in vitro gene transfer. The polyacridine anchor is designed to be optimized for either in vitro or in vivo gene transfer by controlling the binding affinity to DNA and charge of resulting DNA polyplexes. Furthermore, the Cys residue allows coupling of fusogenic peptides, ligands, and PEG. These attributes should allow polyacridine several unique advantages compared to other polycationic DNA binding peptides and polymers.

ACKNOWLEDGMENT

We gratefully acknowledge support for this work from the NIH Grant DK066212.

LITERATURE CITED

(1) Mahato, R. I. (1999) Non-viral peptide-based approaches to gene delivery. *J. Drug Targeting 7*, 249–268.
(2) Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic. *J. Biol. Chem. 269*, 12918–12924.
(3) Van Dyke, R. W. (1996) Acidification of lysosomes and endosomes. *Sub-Cell. Biochem. 27*, 331–60.
(4) Ogris, M., Carlisle, R. C., Bettinger, T., and Seymour, L. W. (2001) Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. *J. Biol. Chem. 276*, 47550–47555.
(5) Parente, R. A., Nadasdi, L., Subbarao, N. K., and Szoka, F. C., Jr. (1990) Association of a pH-sensitive peptide with membrane vesicles: role of amino acid sequence. *Biochemistry 29*, 8713–9.
(6) Parente, R. A., Nir, S., and Szoka, F. C., Jr. (1990) Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry 29*, 8720–8.
(7) Wyman, T. B., Nicol, F., Zelphati, O., Scaria, P. V., Plank, C., and Szoka, F. C. (1997) Design, synthesis and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry 36*, 3008–3017.
(8) Gottschalk, S., Sparrow, J. T., Hauer, J., Mims, M. P., Leland, F. E., Woo, S. L. C., and Smith, L. C. (1996) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther. 3*, 448–457.
(9) Chen, C.-P., Kim, J.-S., Steenblock, E., Liu, D., and Rice, K. G. (2006) Gene transfer with poly-melittin peptides. *Bioconjugate Chem. 17*, 1057–62.
(10) Boeckle, S., Wagner, E., and Ogris, M. (2005) C- versus N-terminally linked melittin-polyethylenimine conjugates: the site of linkage strongly influences activity of DNA polyplexes. *J. Gene Med. 7*, 1335–1347.
(11) Boeckle, S., Fahrmeir, J., Roedl, W., Ogris, M., and Wagner, E. (2006) Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J. Controlled Release 112*, 240–8.
(12) Goto, Y., and Hagihara, Y. (1992) Mechanism of the conformational transition of melittin. *Biochemistry 31*, 732–8.

(13) Subbalakshmi, C., Nagaraj, R., and Sitaram, N. (1999) Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analog with improved antibacterial activity. *FEBS Lett. 448*, 62–6.

(14) Terwilliger, T. C., and Eisenberg, D. (1982) The structure of melittin. II. Interpretation of the structure. *J. Biol. Chem. 257*, 6016–22.

(15) Legendre, J. Y., Bohrmann, T. B., Deuschle, U., Kitas, E., and Supersaxo, A. (1997) Dioleoymelittin as a novel serum-insensitive reagent for efficient transfections of mammalian cells. *Bioconjugate Chem. 8*, 57–63.

(16) Rozema, D. B., Ekena, K., Lewis, D. L., Loomis, A. G., and Wolff, J. A. (2003) Endosomolysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules. *Bioconjugate Chem. 14*, 51–7.

(17) Read, M. L., Logan, A., and Seymour, L. W. (2005) Barriers to gene delivery using synthetic vectors, in *Advances in Genetics* (Hall, J. C., Dunlap, J. C., Friedmann, T., and van Heyningen, V., Eds.) pp 19−46, Academic Press, London.

(18) Dash, P. R., Read, M. L., Barrett, L. B., Wolfert, M. A., and Seymour, L. W. (1999) Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. *Gene Ther. 6*, 643–650.

(19) Burke, R. S., and Pun, S. H. (2008) Extracellular barriers to in vivo PEI and PEGylated PEI polyplex-mediated gene delivery to the liver. *Bioconjugate Chem. 19*, 693–704.

(20) Kwok, K. Y., McKenzie, D. L., Evers, D. L., and Rice, K. G. (1999) Formulation of highly soluble poly(ethylene glycol)-peptide DNA condensates. *J. Pharm. Sci. 88*, 996–1003.

(21) Kwok, K. Y., Park, Y., Yongsheng, Y., McKenzie, D. L., and Rice, K. G. (2003) In vivo gene transfer using sulfhydryl crosslinked PEG-peptide/glycopeptide DNA co-condensates. *J. Pharm. Sci. 92*, 1174–1185.

(22) Chen, C. p., Kim, J. s., Liu, D., Rettig, G. R., McAnuff, M. A., Martin, M. E., and Rice, K. G. (2007) Synthetic PEGylated glycoproteins and their utility in gene delivery. *Bioconjugate Chem. 18*, 371–378.

(23) Ueyama, H., Takagi, M., Waki, M., and Takenaka, S. (2001) DNA binding behavior of peptides carrying acridinyl units: First example of effective poly-intercalation. *Nucleic Acids Symp. Ser. (Oxf.) 1*, 163–164.

(24) Haensler, J., and Szoka, J. F. C. (1993) Synthesis and characterization of a trigalactosylated bisacridine compound to target DNA to hepatocytes. *Bioconjugate Chem. 4*, 85–93.

(25) Shiraishi, T., Hamzavi, R., and Nielsen, P. E. (2005) Targeted delivery of plasmid DNA into the nucleus of cells via nuclear localization signal peptide conjugated to DNA intercalating bis- and trisacridines. *Bioconjugate Chem. 16*, 1112–1116.

(26) Boulanger, C., Di Giorgio, C., and Vierling, P. (2005) Synthesis of acridine-nuclear localization signal (NLS) conjugates and evaluation of their impact on lipoplex and polyplex-based transfection. *Eur. J. Med. Chem. 40*, 1295–306.

(27) Tung, C., Zhu, T., Lackland, H., and Stein, S. (1992) An acridine amino acid derivative for use in Fmoc peptide synthesis. *Peptide Research 5*, 115–8.

(28) Rich, D. H., Gesellchen, P. D., Tong, A., Cheung, A., and Buckner, C. K. (1975) Alkylating derivatives of amino acids and peptides. Synthesis of N-maleoylamino acids, [1-(N-maleoylglycyl)cysteinyl]oxytocin, and [1-(N-maleoyl-11-aminoundecanoyl) cysteinyl]oxytocin. Effects on vasopressin-stimulated water loss from isolated toad bladder. *J. Med. Chem. 18*, 1004–1010.

(29) Wadhwa, M. S., Collard, W. T., Adami, R. C., McKenzie, D. L., and Rice, K. G. (1997) Peptide-mediated gene delivery: influence of peptide structure on gene expression. *Bioconjugate Chem. 8*, 81–8.

(30) Bezanilla, M., Drake, B., Nudler, E., Kashlev, M., Hansma, P. K., and Hansma, H. G. (1994) Motion and enzymatic degreadation of DNA in the atomic force microscope. *Biophys. J. 67*, 2454–9.

(31) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65*, 55–63.

(32) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujumoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150*, 76–85.

(33) Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. (2000) Network protein sequence alignment. *TIBS 25*, 147–150.

(34) Legendre, J. Y., and Szoka, F. C., Jr. (1993) Cyclic amphipathic peptide-DNA complexes mediate high-efficiency transfection of adherent mammalian cells. *Proc. Natl. Acad. Sci. U.S.A. 90*, 893–7.

(35) Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. U.S.A. 89*, 7934–7938.

(36) Midoux, P., Kichler, A., Boutin, V., Maurizot, J. C., and Monsigny, M. (1998) Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjugate Chem. 9*, 260–267.

(37) Schuster, M. J., Wu, G. Y., Walton, C. M., and Wu, C. H. (1999) Multicomponent DNA carrier with a vesicular stomatitis virus G-peptide greatly enhances liver-targeted gene expression in mice. *Bioconjugate Chem. 10*, 1075–1083.

(38) Nishikawa, M., Yamauchi, M., Morimoto, K., Ishida, E., Takakura, Y., and Hashida, M. (2000) Heptocyte-targeted *in vivo* gene expression by intraveneous injection of plasmid DNA complexed with synthetic multi-functional gene delivery system. *Gene Ther. 7*, 548–555.

(39) Rittner, K., Benavente, A., Bompard-Sorlet, A., Heitz, F., Divita, G., Brasseur, R., and Jacobs, E. (2002) New basic membrane-destabilizing peptides for plasmid-based gene delivery in vitro and in vivo. *Mol. Ther. 5*, 104–14.

(40) Li, W., Nicol, F., and Szoka, F. C., Jr. (2004) GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Adv. Drug Delivery Rev. 56*, 967–985.

(41) Collard, W. T., Yang, Y., Kwok, K. Y., Park, Y., and Rice, K. G. (2000) Biodistribution, metabolism, and in vivo gene expression of low molecular weight glycopeptide polyethylene glycol peptide DNA co-condensates. *J. Pharm. Sci. 89*, 499–512.

(42) McKenzie, D. L., Kwok, K. Y., and Rice, K. G. (2000) A potent new class of reductively activated peptide gene delivery agents. *J. Biol. Chem. 275*, 9970–9977.

(43) Park, Y., Kwok, K. Y., Boukarim, C., and Rice, K. G. (2002) Synthesis of sulfhydryl crosslinking poly (ethylene glycol) peptides and glycopeptides as carriers for gene delivery. *Bioconjugate Chem. 13*, 232–239.

(44) Read, M. L., Bremner, K. H., Oupicky, D., Green, N. K., Searle, P. F., and Seymour, L. W. (2003) Vectors based on reducible polycations facilitate intracellular release of nucleic acids. *J. Gene Med. 5*, 232–45.

(45) Zhou, Q.-H., Wu, C., Manickam, D., and Oupický, D. (2009) Evaluation of pharmacokinetics of bioreducible gene delivery vectors by real-time PCR. *Pharm. Res. 26*, 1581–1589.

(46) Meyer, M., Philipp, A., Oskuee, R., Schmidt, C., and Wagner, E. (2008) Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery. *J. Am. Chem. Soc. 130*, 3272–3.

BC9003124