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Comparative gene transfer efficiency of low molecular weight polylysine DNA-condensing peptides

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Abstract: In a previous report (M.S. Wadhwa *et al.* (1997) *Bioconjugate Chem.* **8,** 81–88), we synthesized a panel of polylysine-containing peptides and determined that a minimal repeating lysine chain of 18 residues followed by a tryptophan and an alkylated cysteine residue ($AlkCWK_{18}$) resulted in the formation of optimal size (78 nm diameter) plasmid DNA condensates that mediated efficient *in vitro* gene transfer. Shorter polylysine chains produced larger DNA condensates and mediated much lower gene expression while longer lysine chains were equivalent to $AlkCWK_{18}$. Surprisingly, $AlkCWK_{18}$ (molecular weight 2672) was a much better gene transfer agent than commercially available low molecular weight polylysine (molecular weight 1000–4000), despite its similar molecular weight. Possible explanations were that the cysteine or tryptophan residue in $AlkCWK_{18}$ contributed to the DNA binding and the formation of small condensates or that the homogeneity of $AlkCWK_{18}$ relative to low molecular weight polylysine facilitated optimal condensation. To test these hypotheses, the present study prepared $AlkCYK_{18}$ and K_{20} and used these to form DNA condensates and conduct *in vitro* gene transfer. The results established that DNA condensates prepared with either $AlkCYK_{18}$ or K_{20} possessed identical particle size and mediated *in vitro* gene transfer efficiencies that were indistinguishable from $AlkCWK_{18}$ DNA condensates, eliminating the possibility of contributions from cysteine or tryptophan. However, a detailed chromatographic and electrospray mass spectrometry analysis of low molecular weight polylysine revealed it to possess a much lower than anticipated average chain length of dp 6. Thus, the short chain length of low molecular weight polylysine explains its inability to form small DNA condensates and mediate efficient gene transfer relative to

AlkCWK$_{18}$ DNA condensates. These experiments further emphasize the need to develop homogenous low molecular weight carrier molecules for nonviral gene delivery.

Abbreviations: DIC, diisopropylcarbodiimide; DTT, dithiothreitol; EDT, ethanedithiol; ES-MS, electrospray mass spectrometry; FBS, fetal bovine serum; HBM, Hepes-buffered mannitol; HOBt, 9-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; LB, Lurai-Bertani; LMW, low molecular weight; MEM, minimal essential media; MW, molecular weight; NMR, nuclear magnetic resonance; RLU, relative light units; SFM, serum-free media; TFA, trifluoroacetic acid.

Introduction

A variety of nonviral gene transfer agents have been developed as carriers that mediate the *in vitro* and *in vivo* transfer of foreign DNA. These generally fall into the three categories of cationic lipids (1), synthetic polymers (including dendrimers, polyethylenimine and peptiods) (2–5) and peptides (6–8).

Among the peptides studied as gene delivery agents, polylysine has been shown to bind electrostatically to DNA and form condensates in which the peptide length influences particle size and the resulting gene transfer efficiency (9, 10). One study found that condensed DNA particle size decreased with decreasing polylysine chain length such that 4 kDa polylysine produced 20–30 nm DNA condensates whereas 224 kDa polylysine resulted in 120–300 nm DNA condensates (9). A more recent study reported that 27 kDa polylysine forms 2-μm diameter particles that are poor mediators of gene transfer relative to other cationic polymers (10).

A further complication arises during the covalent attachment of effector molecules to polylysine, such as carbohydrates, proteins or polyethylene glycol, to prepare DNA carrier molecules capable of achieving cell type selective targeting (11–15). The chemical derivatization of polydisperse polylysine further increases the heterogeneity of these carrier molecules, contributing to the variable performance of the resulting DNA carrier formulation (16). Consequently, homogenous peptide carriers have the advantage of precise chemical control during the synthesis of conjugates and formulation with plasmid DNA resulting in greater reproducibility during *in vitro* and *in vivo* gene transfer.

We have previously synthesized DNA-condensing peptides composed of cysteine, tryptophan and 3–36 residues of lysine in an attempt to define the minimal polylysine chain length needed to mediate efficient gene transfer (17). According to this design, the cysteine residue served as a specific functional group for ligand attachment and the tryptophan afforded a hydrophobic chromophore to aid in high-performance liquid chromatography (HPLC) purification. Detailed analysis of these peptides established that a continuous sequence of 18 lysines was sufficient to condense plasmid DNA into 78 nm particles, whereas shorter polylysine chains exhibited weaker affinity and resulted in larger (200–2000 nm) DNA condensates which were inferior in mediating *in vitro* gene expression (17). During the course of this study, we found that commercially available low molecular weight (LMW) polylysine (1000–4000 Da) formed much larger condensates and failed to mediate significant gene expression. It was suggested that, in addition to polylysine chain length, the insertion of an aromatic amino acid may contribute to the DNA-binding affinity and the formation of small condensates through intercalation.

Several studies have previously observed enhanced peptide binding to DNA through intercalation of aromatic amino acid residues (18–20). NMR experiments demonstrated that a synthetic peptide (YSPTSPSY) positioned two tyrosine residues to simultaneously intercalate with DNA to enhance its binding affinity (18). Likewise, the chirality and position of an aromatic amino acid within a peptide was also found to influence the intercalation and the binding affinity to DNA (19). Furthermore, the number of aromatic amino acids correlated with the degree of intercalation into DNA such that peptides containing > 30% aromatic amino acids had higher affinity for DNA than polylysine (20).

In the present study we correlated the relative DNA-binding affinity, particle size and the resulting gene transfer efficiency for several homogenous peptides with and without aromatic residues. The results rationalize our earlier observations on LMW polylysine and help to further establish the optimal size and composition of DNA-condensing peptides for nonviral gene delivery.

Experimental Procedures

N-terminal Fmoc-protected amino acids, and all other reagents for peptide synthesis, were obtained from Advanced ChemTech (Lexington, KY, USA). Minimum essential media (MEM), dithiothreitol (DTT), iodoacetic acid and LMW polylysine (MW 1000–4000, Lot #14H5526) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanedithiol (EDT) and N,N-dimethylformamide were

purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Luria-Bertani (LB) media, LB agar, D-luciferin and luciferase from *Photinus pyralis* (EC1.13.12.7) were obtained from Boehringer-Mannheim (Indianapolis, IN, USA). HepG2 cells were from the American Type Culture Collection (Rockville, MD, USA). Inactivated 'qualified' fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA) and thiazole orange was a gift from Beckton-Dickinson Immunocytometry Systems (San Jose, CA, USA). The 5.6 kb plasmid pCMLV encoding the reporter gene luciferase under the control of the cytomegalovirus promoter was a gift from Dr M. A. Hickman at the University of California, Davis (21). Peptide synthesis was performed on a computer-interfaced Model 90 Advanced ChemTech solid-phase peptide synthesizer (Lexington, KY, USA). Peptide purification was performed using a semipreparative (10 μm) C_{18} reverse phase (RP)-HPLC column and cation exchange chromatography was performed using an analytical (5 μm) 400VHP575 cationic exchange column from Vydac (Hesperia, CA, USA). HPLC was performed using a computer-interfaced HPLC and fraction collector from ISCO (Lincoln, NE, USA).

Peptide synthesis and characterization

CWK_{18}, CYK_{18} and K_{20} were synthesized using standard Fmoc procedures with 9-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) couplings followed by N-capping with acetic anhydride after each round of coupling. The N-terminal Fmoc group was purposely left on K_{20} at the completion of the synthesis to enhance purification (22). Peptides were cleaved from the resin and side-chain protecting groups were removed by 1 h reaction with TFA : EDT : water (95 : 2.5 : 2.5 v/v/v) for the cysteine-containing peptides and TFA : water (95 : 5 v/v) for K_{20}. CWK_{18} was purified as described previously (17). CYK_{18} was purified by injecting 3 μmol onto a Vydac C_{18} RP-HPLC column (2 × 25 cm) eluted at 10 mL/min with 0.1% TFA and a gradient of 5–20% acetonitrile over 30 min while monitoring absorbance at 274 nm. The major peptide peak eluting at 21 min was collected and pooled from multiple runs, concentrated by rotary evaporation, lyophilized and stored dry at – 20°C. The purified peptide was dissolved in 0.1% TFA (degassed with N_2) and quantified by absorbance (CYK_{18}; ε_{280nm} = 1330 M^{-1}/cm) to determine the isolated yield which was typically 40%.

CWK_{18} and CYK_{18} were alkylated with iodoacetic acid by adding 50 μmol of dithiothreitol and 250 μmol of iodoacetic acid to 2.5 μmol of peptide in 1.5 mL of 50 mM Tris (pH 7.5) buffer and reacted at room temperature for 1 h. Each alkylated peptide was purified on RP-HPLC resulting in > 95% purity and then analyzed by electrospray mass spectroscopy (ES-MS) by infusing a 5-nmol/mL solution of peptide prepared in 50 : 46 : 4% (v/v) acetonitrile : water : acetic acid into the source of a VG (Micromass) Platform single quadrapole mass spectrometer (0–3000 m/z) operated in the positive ion mode. A mass of $2671.4^{obs}/2670.8^{calc}$ and $2650.1^{obs}/2649.5^{calc}$ was determined for (alkylated) $AlkCWK_{18}$ and (alkylated) $AlkCYK_{18}$, respectively.

$Fmoc-K_{20}$ was purified by injecting 3 μmol onto a semipreparative Vydac C_{18} RP-HPLC column (2 × 25 cm) eluted isocratically at 10 mL/min with 16% acetonitrile while detecting by absorbance at 260 nm. The product peak eluting at 23 min was collected and concentrated to dryness. The N-terminal Fmoc was removed by reacting the peptide with 5% v/v triethylamine for 6 h at room temperature (22). Following ether extraction, the peptide was purified to homogeneity on preparative HPLC by injecting 3 μmol onto a semipreparative RP-HPLC column eluting isocratically at 10 mL/min with 0.05% TFA and 5% acetonitrile while monitoring absorbance at 214 nm. The product peak eluting at 22 min was pooled from multiple runs, dried and dissolved in 0.1% TFA. The purity of K_{20} was also > 95% as determined from cation exchange HPLC analysis as described below. The concentration of a K_{20} stock solution was determined by fluorescamine analysis (23) using $AlkCWK_{18}$ as a primary standard which determined the yield of K_{20} from the synthesis to be 69%. The mass of K_{20} was determined to be $2583.0^{obs}/2581.4^{calc}$ by ES-MS as described above.

The concentration of a stock solution of LMW polylysine (1000–4000, HBr salt form) was also established using $AlkCWK_{18}$ as a calibrator for fluorescamine analysis. LMW polylysine was analyzed by injecting 12 nmol onto a cation-exchange column (Vydac 400 V HP575) eluted at 1 mL/min with a sodium chloride gradient of 0.1–1.5 M over 30 min while detecting the eluting peaks by absorbance at 214 nm. The chromatographic drift resulting from gradient elution was corrected by subtracting a blank.

DNA condensation and gene expression

Peptide DNA condensates were prepared at a final DNA concentration of 20 μg/mL and at a peptide/DNA stoichiometry varying from 0.1 to 1.5 nmol of peptide per μg of DNA

(0.5 : 1 to 8 : 1 amine : phosphate ratio). The condensates were formed by adding peptide (2–30 nmol) prepared in 500 μL of isotonic Hepes-buffered mannitol (HBM, 0.27 M mannitol, 5 mM sodium Hepes, pH 7.5) to 20 μg of DNA in 500 μL HBM while vortexing, followed by equilibration at room temperature for 30 min.

Peptide binding to DNA was monitored by a fluorescence titration assay (24). A 1-μg aliquot of the peptide DNA condensate was diluted to 1 mL in HBM containing 0.1 μM thiazole orange. The fluorescence of the intercalated dye was measured on an LS50B fluorimeter (Perkin-Elmer, UK) in a microcuvette by exciting at 500 nm while monitoring emission at 530 nm, with the slits set at 15 and 20 nm and photomultiplier gain set to 700 V. DNA condensation was monitored by measuring total scattered light at 90° by setting both monochromators to 450 nm and decreasing slit widths to 2.5 nm. Fluorescence and scattered light intensity blanks were subtracted from all values before data analysis.

Particle size analysis was measured for peptide DNA condensates prepared at a DNA concentration of 20 μg/mL in HBM and at a stoichiometry of 0.8 nmol of peptide per μg of DNA (5.5 : 1 amine : phosphate). Samples were analyzed using a Brookhaven ZetaPlus quasielastic light-scattering particle sizer. The mean diameter and population distribution were computed from the diffusion coefficient using functions supplied by the instrument.

HepG2 cells (2×10^6 cells) were plated on 6×35 mm wells and grown to 40–70% confluency in MEM supplemented with 10% FBS, penicillin and streptomycin (10 000 U/mL), sodium pyruvate (100 mM) and L-glutamine (200 mM). Transfections were performed in MEM (2 mL per 35 mm well) with 2% FBS, with 80 μM chloroquine (25). Peptide DNA condensates (10 μg of DNA in 0.5 mL HBM) were added dropwise to triplicate wells. After 5 h incubation at 37°C, the media was replaced with MEM supplemented with 10% FBS.

Luciferase expression was determined at 24 h using some modification of a published method (26). Cells were washed twice with ice-cold phosphate-buffered saline (calcium and magnesium free) and then treated with 0.5 mL of ice-cold lysis buffer (25 mM Tris chloride pH 7.8, 1 mM EDTA, 8 mM magnesium chloride, 1% Triton X-100, 1 mM DTT) for 10 min. The cell lysate was scraped, transferred to 1.5 mL micro-centrifuge tubes and centrifuged for 7 min at 13 000 **g** at 4°C to pellet debris.

Lysis buffer (300 μL), sodium-ATP (4 μL of a 180-mM solution, pH 7, 4°C) and cell lysate (100 μL, 4°C) were combined in a test tube, briefly mixed and immediately placed in the luminometer. Luciferase relative light units (RLU) were recorded on a Lumat LB 9501 (Berthold Systems, Germany) with 10 s integration after automatic injection of 100 μL of 0.5 mM D-luciferin (prepared fresh in lysis buffer without DTT). The RLU were converted into fmol using a standard curve generated by adding a known amount of the luciferase (0.01–100 fmol with specific activity of 2.5 nU/fmol) to 35 mm wells containing 40–70% confluent HepG2 cells. The cells were processed as described above resulting in a standard curve with an average slope of 220 000 RLU/fmol of enzyme. Protein concentrations were measured by Bradford assay using bovine serum albumin as a standard (27). The amount of luciferase recovered in each sample was normalized to milligrams of protein and the mean and standard deviation obtained from each triplicate are reported.

LipofectAce™ (Gibco BRL, 1 : 2.5 w/w dimethyl dioctadecylammonium bromide and dioleoyl phosphatidylethanolamine) was used to mediate gene transfection according to the manufacturer's instructions. The ratio of DNA to lipofectAce was optimized for HepG2 cells. An optimal DNA/lipofectAce ratio was achieved by dissolving 10 μg of DNA in 100 μL of serum-free media (SFM) followed by adding 60 μL of lipofectAce prepared in 140 μL of SFM. The LipofectAce–DNA complex was then diluted with 1.7 mL of SFM and used to transfect HepG2 cells for 5 h followed by replacing the transfecting media with supplemented 10% FBS. The cells were incubated for a total of 24 h then harvested and analyzed for luciferase as described above.

Results and Discussion

Solid-phase synthesis of polylysine-containing peptides is complicated by poor yields arising from the repeating sequence and by difficulty in purification of the desired full-length peptide. These problems can be minimized through double coupling, end-capping, and by positioning a hydrophobic aromatic amino acid near the N-terminus to allow peak identification on RP-HPLC, strategies that proved successful in the synthesis of CWK_{18}.

Consequently, substitution of tryptophan with tyrosine to prepare CYK_{18} (Fig. 1) was of no greater synthetic challenge than the synthesis of CWK_{18} and was viewed as a first step toward determining whether tryptophan enhanced the binding of CWK_{18} to DNA. A difference in the DNA intercalation of aromatic residue in $AlkCYK_{18}$ and $AlkCWK_{18}$ could lead to differences in binding affinity and the resulting DNA condensate particle size. However, a detailed comparison of the fluorescence quench and total
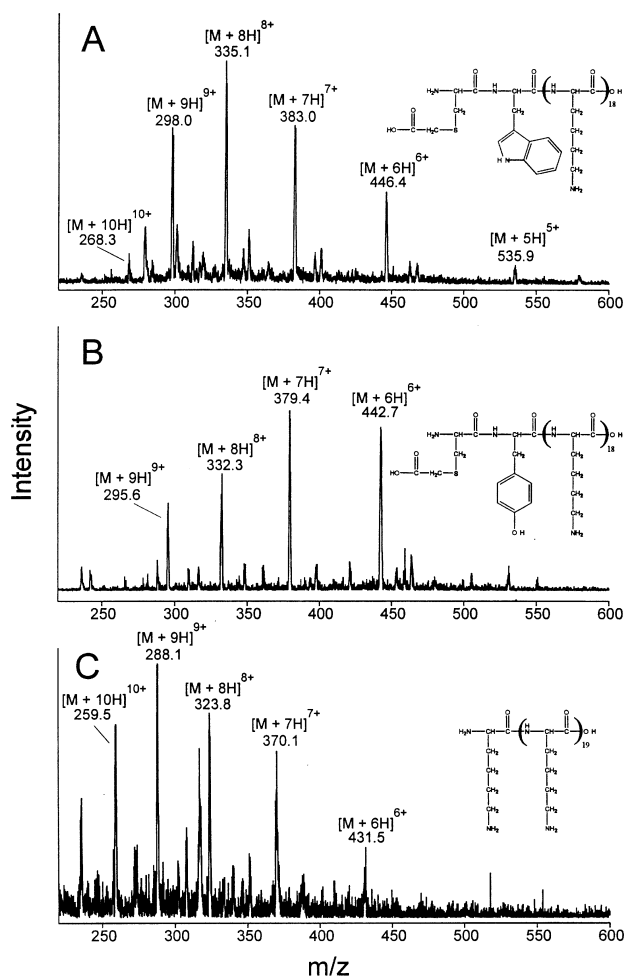
Figure 1. Structure of polylysine peptides used for gene delivery. The structure and ES-MS analysis for $AlkCWK_{18}$, $AlkCYK_{18}$ and K_{20} are compared. Each peptide was detected as the $[M + 6H]^{6+}$–$[M + 9H]^{9+}$ charge state.

Figure 2. Analysis of DNA condensation with polylysine peptides. The thiazole orange fluorescence intensity (open symbol) is plotted along with the total light scattering (closed symbol) for the titration of DNA with $AlkCWK_{18}$ (A), $AlkCYK_{18}$ (B) K_{20} (C) and LMW polylysine (D). The QELS particle size analysis for DNA peptide condensates prepared at a concentration of 0.8 nmol of peptide per μg of DNA (charge ratio $\approx 5.5 : 1$) is shown for $AlkCWK_{18}$ (A'), $AlkCYK_{18}$ (B') K_{20} (C') and LMW polylysine (D').

light-scattering titration curves for $AlkCWK_{18}$ and $AlkCYK_{18}$ indicated no significant difference in their binding to DNA (Fig. 2A, B). Each peptide fully displaced the intercalating dye at a 1.1 : 1 amine : phosphate ratio. The light-scattering profiles established that each condensate was fully formed at stoichiometries above a 2.5 : 1 amine : phosphate ratio. Likewise, QELS analysis of $AlkCWK_{18}$ and $AlkCYK_{18}$ DNA condensates indicated that each formed a major (> 95%) population of particles with a mean diameter of 43 nm (Fig. 2A', B'). Thus, the physical properties of $AlkCWK_{18}$ and $AlkCYK_{18}$ DNA condensates were indistinguishable.

$AlkCWK_{18}$ and $AlkCYK_{18}$ DNA condensates were also compared for *in vitro* gene transfer in HepG2 cells. Both mediated essentially equivalent levels of gene expression in the presence of chloroquine and serum which was slightly higher than that mediated by LipofectAce in the absence of chloroquine and serum (Fig. 3). These results suggested that
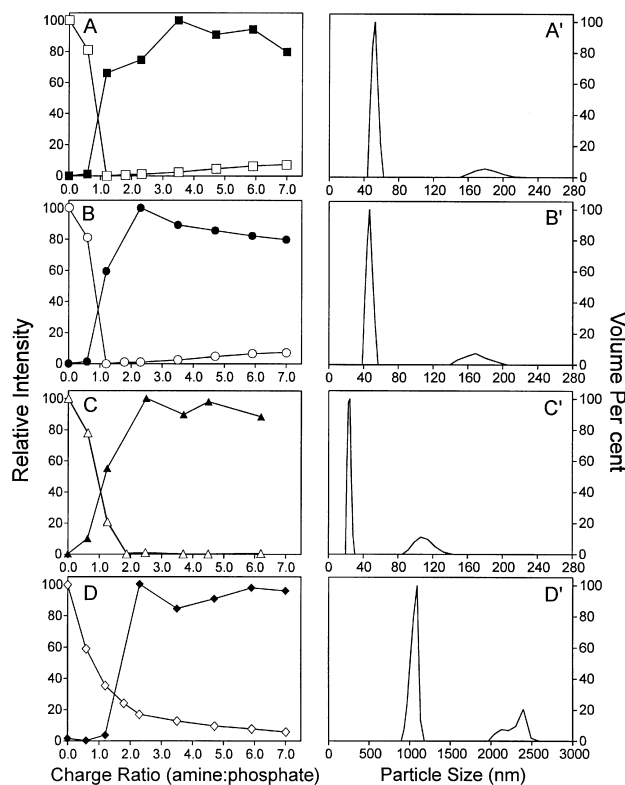
either the aromatic residue in $AlkCWK_{18}$ and $AlkCYK_{18}$ function equivalently or that these residues do not significantly influence peptide binding, condensation and gene transfer efficiency of DNA.

The further comparison of the properties of homogenous polylysine DNA-condensing peptides required the removal of the aromatic residue to prepare K_{20}. However, this complicated the isolation of the full-length peptide due to the lack of hydrophobic chromophore during HPLC purification. Consequently, this obstacle was overcome while synthesizing K_{20} by retaining Fmoc on the N-terminus during the final cycle of synthesis, thereby using it as removable chromophore following RP-HPLC purification.

Comparison of the fluorescence and total light-scattering titration for K_{20} and commercially available LMW polylysine established that K_{20} completely quenched the fluorescence at an amine : phosphate ratio of $\approx 2 : 1$, while LMW polylysine bound to DNA over a broader range and only reached an equivalent point at an amine : - phosphate ratio of $\approx 4 : 1$ (Fig. 2C, D). In contrast, the total
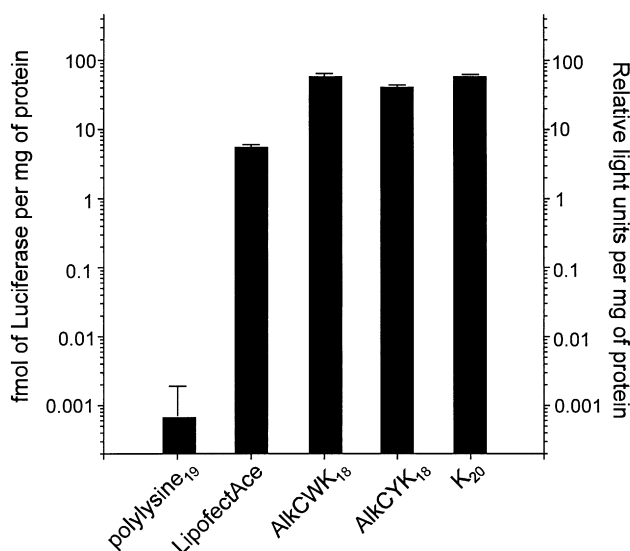
Figure 3. Gene transfer efficiency of peptide DNA condensates. The gene transfer efficiency, as determined by luciferase expression in HepG2 cells, is compared for peptide DNA condensates prepared at 0.8 nmol of peptide per μg of DNA (charge ratio of $\approx$ 5.5 : 1) for the peptides indicated. LipofectAce transfection is included for comparison.

Figure 4. Cation-exchange HPLC and ES-MS analysis of LMW polylysine. The polydispersity of LMW polylysine was determined using cation-exchange HPLC, which includes a spiked sample of K_{20} as indicated in (A). ES-MS analysis of the major LMW polylysine peak established it as dp 6 as illustrated in (B). The results confirmed an average chain length of 6 for LMW polylysine.

light-scattering profile for K_{20} and LMW polylysine indicated that each formed fully condensed DNA at an amine : phosphate ratio above 3 : 1.

The most significant difference between K_{20} and LMW polylysine was the particle size of their DNA condensates. K_{20} formed small DNA condensates of $\approx$ 32 nm diameter (Fig. 2C') which were comparable with those formed by $AlkCWK_{18}$ and $AlkCYK_{18}$ (Fig. 2A', B'), whereas LMW polylysine formed large DNA condensates that were > 1 μm in diameter (Fig. 2D').

K_{20} and LMW polylysine DNA condensates were also compared for their ability to transform cells *in vitro*. LMW polylysine demonstrated a four-order of magnitude decrease in mediating gene transfer in HepG2 cells relative to K_{20}, which was equivalent to the gene transfer efficiency mediated by $AlkCWK_{18}$ and $AlkCYK_{18}$ (Fig. 3). From these results, we concluded that differences in amino acid composition cannot account for the gene transfer properties of $AlkCWK_{18}$, $AlkCYK_{18}$ and K_{20} relative to LMW polylysine.

ES-MS molecular weight analysis provided definitive proof of the structure of $AlkCWK_{18}$, $AlkCYK_{18}$ and K_{20} (Fig. 1) but proved unsuccessful for characterizing the mass of LMW polylysine. Therefore, cation-exchange chromatography was used to separate LMW polylysine into its component peptides (Fig. 4A). Coelution of K_{20} with LMW polylysine not only established the polydispersity of the later, but also revealed its average molecular weight to be significantly lower than dp 20, since polylysines are retained
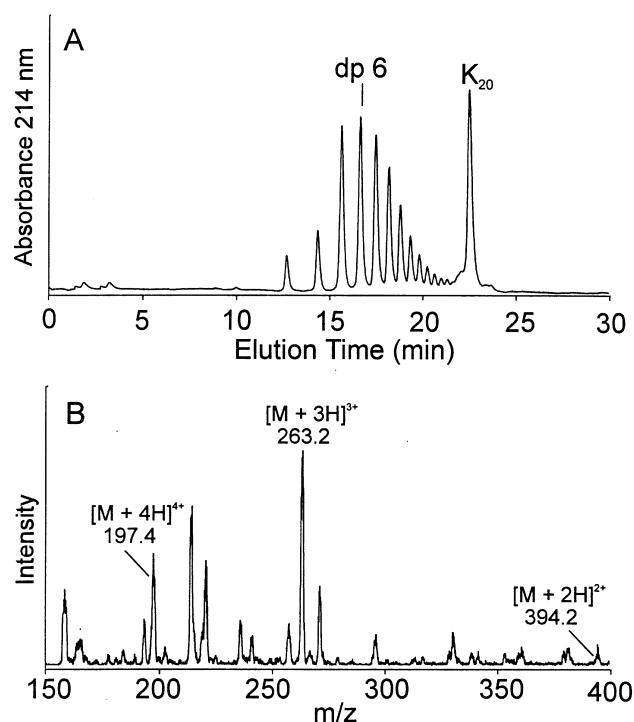
according to their number of cationic charges. This was confirmed by isolating the major LMW polylysine peptide which produced an ES-MS mass of 786.5 confirming it as dp 6 (Fig. 4B). A 10% impurity of dp 6 produced an ion $[M + 3H]^{3+}$ at *m/z* of 220.3 predicting a mass of 658.2 corresponding to dp 5. Since the molar absorptivity of each polylysine peptide is dependent on the number of lysine residues, the cation-exchange chromatograph represents a weight average distribution analysis of the polypeptide mixture. Thereby, dp 5, 6 and 7 are the major peptides based both on a weight and mol basis.

The conclusion drawn from this study is that a peptide of $\approx$ 18 lysines is sufficient to condense DNA into small particles that mediate efficient gene transfer in the presence of chloroquine. It appears that a single aromatic amino acid does not play a significant role in the ability of $AlkCWK_{18}$ and $AlkCYK_{18}$ to bind and condense DNA. However, LMW polylysine does not adequately substitute for $AlkCWk_{18}$, $AlkCYK_{18}$ or K_{20} because it possesses significant heterogeneity and has a mean size distribution weighted towards lower molecular weight polylysine chains which condense DNA to form large particles. This is consistent with our previous results, which demonstrated that LMW polylysine

peptides (degree of polymerization (dp) 10) condense DNA, but form large particles that are > 1 μm in size (17).

The size of DNA condensates appears to be directly correlated to the gene transfer efficiency. However, an alternative explanation is that the relative serum stability of the condensed DNA also correlates with the gene transfer efficiency. We have recently reported that weak binding peptides possessing eight lysine residues (AlkCWK$_8$) dissociate from DNA at sodium chloride concentrations below 0.15 M (normal saline) and fail to protect DNA from endonuclease attack in serum (28). Thus, the inefficient gene transfer mediated by DNA condensates prepared with LMW polylysine could also be due to premature metabolism of the DNA in the cell culture media during *in vitro* transfection. Consequently, AlkCWK$_{18}$ is a homogenous peptide that produces both small condensates and protects DNA from degradation in serum (26). These are important attributes towards the development of homogenous nonviral gene delivery agents. Future studies will demonstrate the utility of functionalizing the cysteine residue with targeting ligands to create homogenous LMW carrier molecules for gene delivery.

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