Design and Biophysical Characterization of Novel Polycationic ε-Peptides for DNA Compaction and Delivery

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Design and solid-phase synthesis of novel and chemically defined linear and branched ε-oligo(l-lysines) (denoted ε-Kn, where n is the number of lysine residues) and their α-substituted homologues (ε-(R)K10, ε-(Y)K10, ε-(L)K10, ε-(YR)K10, and ε-(LYR)K10) for DNA compaction and delivery are reported. The ability to condense viral (T2 and T4) and plasmid DNA as well as the size of ε-peptide DNA complexes under different conditions was investigated with static and dynamic light scattering, isothermal titration calorimetry, and fluorescence microscopy. Nanoparticle diameters varied from 100 to 150 and 375 to 550 nm for plasmid and T4 DNA peptide complexes, respectively. Smaller sizes were observed for oligo(l-lysines) compared to α-poly(l-lysine). The linear ε-oligo-lysines are less toxic and ε-(LYR)K10 showed higher transfection efficiency in HeLa cells than corresponding controls. The results also demonstrate that with a branched design having pendant groups of short α-oligopeptides, improved transfection can be achieved. This study supports the hypothesis that available α-oligolysine derived systems would potentially have more favorable delivery properties if they are based instead on ε-oligo(l-lysines). The flexible design and unambiguous synthesis that enables variation of pendant groups holds promise for optimization of such ε-peptides to achieve improved DNA compaction and delivery.

Introduction

As a highly negatively charged polyelectrolyte, DNA adopts an extended random coil conformation in dilute solutions which is driven by repulsion between neighboring segments and entropic gain of flexibility. However, in the presence of polycationic agents, like poly(L-lysine), polyamines, polypeptides, and synthetic polymers, like polyethyleneimine, monomolecular compaction to nanoparticles or intermolecular DNA aggregation will be induced.1–3 Monomolecular collapse occurs at micro-compactation to nanoparticles or intermolecular DNA aggregation and synthetic polymers, like polyethylenimine, monomolecular polycationic peptides and in particular systems derived from uptake of the DNA.4 These vectors condense the negatively charged DNA molecule or liposome bilayers formed by lipids with cationic headgroups.5–9 Considerable enhancement of transfection has been demonstrated by DNA compaction induced by liposome vectors where high molecular weight polycationic peptides such as polylysine or protamine have been added.10–12 Given these favorable physical and biological properties, we reasoned that controlled and unambiguous solid-phase synthesis of ε-peptides, designed in a flexible manner, can form the basis for a novel type of cationic peptide DNA compaction agents that can be explored for use as DNA compaction agent with potential applications for nonviral gene delivery. In this work we describe the design and synthesis of ε-oligo(l-lysines) and their branched α-amino-substituted derivatives with defined lengths and composition. The focus of the present work is on the design, synthesis, and biophysical characterization of DNA compaction for these novel peptides. We also performed some preliminary evaluation of cytotoxicity and transfection efficacy in two cell lines, HeLa and 293F cells. In this work we show that linear ε-oligo(l-lysines) produced smaller DNA condensates and are associated with lower cytotoxicity and better in vitro transfection than the corresponding α-PLL. The results also demonstrate that with a branched design having pendant groups...
of short α-oligopeptides, improved transfection can be achieved. This study supports the hypothesis that available α-oligolysine derived systems would potentially have more favorable delivery properties if they are based instead on ε-oligo(L-lysines), motivating further optimization of design and more detailed investigations under varied biological conditions.

Materials and Methods

DNA. Highly purified coliphage T2 DNA, 164 kilobase pairs (kbp), was purchased from Sigma-Aldrich (St. Louis, MO). DNA was dissolved in Tris buffer (5 mM Tris, pH 7.3). The concentration of the stock solution was adjusted to 0.5 mg/mL. The stock solution was further diluted in 1 mM Tris buffer to give a concentration of 2 µg/mL. Bacteriophage T4GT7 DNA, 166 kilobase pairs, was purchased from Nippon Gene (Toyama, Japan). The T4GT7 DNA stock solution is 0.32 µg/µL in 10 mM Tris-HCl (pH 7.9) and 1.0 mM EDTA buffer. Plasmid pEGFP-N1 (4.7 kbp), encoding green fluorescent protein, GFP, was a gift from Lu Yanning at Nanyang Technological University. The plasmid was amplified in E. coli DH5α strain and isolated using QIAGEN (Valencia, CA) HiSpeed Plasmid Purification Giga Kit (plasmid stock solution is 0.9 mg/mL in 10 mM KCl). All DNA concentrations were determined by a Varian Cary 300 Bio UV–visible spectrophotometer (Palo Alto, CA) using an extinction coefficient of 0.0196 mL/(µg·cm) (6600 M⁻¹·cm⁻¹) at 260 nm. The absorbance ratio (A₂₆₀/A₅₄₀) was above 1.8, indicating absence of protein contamination.

Peptides. α-Poly(L-lysine), α-PLL, (Mₙ = 8300 Da, degree of polymerization 40 by low-angle laser light scattering; Mₚ = 14600 Da, degree of polymerization 70 by viscosity) was purchased from Sigma-Aldrich and used without further purification. Two types of peptides have been synthesized; unsubstituted ε-oligo(L-lysines) of varying length and α-substituted ε-oligo(L-lysines) (the notation “L” is henceforth omitted) (Figure 1). α-Poly(L-lysine), α-PLL, with degree of polymerization 31 was purchased from Chisso Corporation (Tokyo, Japan). The unsubstituted ε-peptides ε-K₅, ε-K₆, ε-K₇, ε-K₈, ε-K₉, and ε-K₁₀, and the substituted ε-(R)K₁₀, ε-(Y)K₁₀, ε-(L)K₁₀, ε-(YR)K₁₀, and ε-(LYR)K₁₀ were synthesized at Vanderbilt University using solid-phase peptide synthesis. All amino acids and coupling reagents were purchased from Novabiochem (San Diego, CA). The MBHA resin (0.2 mmol/g substitution) was purchased from Advanced ChemTech (Louisville, KY). All solvents used were of the highest commercial grade. The ε-K₅ homologues were constructed on the MBHA resin using Boc-Lys(Fmoc)-OH and Boc-Lys(2-Cl-Z)-OH for protection of the final epsilon amine. They were prepared by Fmoc chemistry using 20% piperidine in DMF for 30 min at the deprotection step. Deprotection of the Boc-α-amines by 50% trifluoroacetic acid in DCM for 30 min generated the free α-amino for coupling in the α-substituted ε-K₁₀ homologues. Each of the coupling steps for ε-K₅ homologues was achieved in 2 h through a combination of coupling reagents, DCC/HOBt, in DMF and DCM (1:1, v/v). However, the α-substituted amino acids on the ε-K₁₀ homologues required a longer time or double coupling for complete coupling reaction. All the coupling steps were confirmed by the Kaiser test. The α-substituted and ε-K₅ homologues were then cleaved from the resin using a standard method of HF (m-meso:HF, 1:10, v/v) for 1 h and for 2–4 h in the case of arginine containing peptides. The crude peptides were purified by preparative RP-HPLC on a Waters 600 system using a C₁₈ Vydac column 22 × 250 mm). All HPLC was carried out using a linear gradient of buffer A (0.05% TFA in H₂O) and buffer B (60% CH₃CN in H₂O with 0.04% TFA). Products were confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using a PerSeptive Biosystems Voyager instrument. Measurements were taken in the linear mode with α-cyano-4-hydroxycinnamic acid as the matrix. The concentration and purity of the ε-oligolysines were checked by HPLC analysis using an analytical C₁₈ column (10 × 250 mm) and a linear gradient of eluent similar to that applied in protein purification (see above). The wavelength of the detector was set to 220 nm. The elution curves did not show the presence of any admixtures or products different from the expected one. The concentrations were calculated from integrated absorbance using the extinction coefficient determined from HPLC data on calibrated peptide solutions. The chemical structures of these polypeptides are given in Figure 1. The peptide stock solutions (5 mg/mL) were prepared in sterile, double-distilled water, and appropriate dilutions were made in 1 mM Tris buffer.

Other Materials. HeLa cells were gifts from Lu Yanning at the School of Biological Sciences of Nanyang Technological University (Singapore). DMEM and PBS and FBS were purchased from Invitrogen (Carlsbad, CA). 293F cells (human kidney embryonic cells) were gifts from Dr. Rupert C. Wilmouth at Nanyang Technological University.

Figure 1. (A) Chemical structure of linear α-oligolysines and ε-oligolysines. (B) Synthetic scheme for solid-phase chemistry preparation of dendrimeric ε-oligolysines α-amino acid derivatives. (L, Y, and R represent amino acid residue leucine, tyrosine, and arginine, respectively.)
(Singapore). DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, 1 µg/µL) solution was purchased from Roche (Basel, Switzerland). Tris, DMSO, MTI, and DAPI were obtained from Sigma-Aldrich. All other chemicals for buffers were purchased from Fisher Scientific (Pittsburgh).

**Static Light Scattering.** Light scattering experiments were performed using a Varian Cary Eclipse fluorescence spectrophotometer by detection of the intensity of the scattered light at 90° to the incident light. The excitation wavelength was set to 350 nm. The condensation experiment was performed in 1 mM Tris buffer, pH 7.3. All buffers were filtered through 0.45 µm filters. Samples were prepared by titrating 3 mL of a solution containing T2 or T4 DNA (2 µg/µL) in the appropriate buffer with the solution containing the peptides. Two different concentrations of peptides were used for titration: 50 and 500 µg/µL; the final peptide concentration in the cuvette was 70 µg/µL. The titration was done in quartz cuvettes which are used as light-scattering cells. The filtration effectively reduces the scattering from dust and impurities. Every point in the figures shown was obtained from three separate experiments with reproducibility within 5%.

**Dynamic Light Scattering (DLS).** Dynamic light scattering (DLS) experiments were conducted using a Brookhaven 90plus particle size analyzer (Long Island, NY). A laser beam was passed through a PMMA cell containing the samples (peptides/DNA mixture; three DNA solutions, 1.3 µg/µL T4 DNA, 70 and 14 µg/µL, plasmid DNA were used, and the final peptide concentration was 70 µg/µL), and the scattered light was measured at a 90° angle to the incident beam. The particle sizes were calculated by applying standard theory connecting light scattering autocorrelation function, translational diffusion coefficient, and particle size using software provided by the equipment manufacturer. Particle sizes are expressed as effective diameter assuming a log-normal distribution.

**Fluorescence Microscopy.** A Nikon Eclipse 90i fluorescence microscope (Tokyo, Japan) equipped with a 100× oil-immersed objective lens was used for studying the conformational change of DNA induced by peptides. DNA molecules were visualized using DAPI according to the technique described in ref 19 (1:1 DNA/DAPI molar ratio was used). To prevent DNA degradation and precipitation onto the glass surface, special care was taken to clean the glass microscope slides and coverslips thoroughly before observations. The cleaning procedure described by Melnikov et al. was applied.19 The microscope slides were soaked in hydrogen peroxide for more than 1 h, rinsed repeatedly with distilled water, and then immersed in ethanol for 1 h and dried at 35 °C for 30 min.19

**Isothermal Titration Calorimetry (ITC).** A VP-ITC microcalorimeter from MicroCal (Northampton, MA) was used at 25.0 °C. The calorimeter was checked and calibrated by applying procedures provided in the device documentation. All DNA and cationic ligand solutions were dissolved in 10 mM KCl and prepared using the same stock solvent. The same plasmid (pEGFP-N1 4.7 kb) as in transfection experiments was used. Stock solutions of the cationic ligands were prepared gravimetrically. At the beginning of titration, concentration of the plasmid DNA in the cell was 0.40 mM DNA phosphate groups (140 µg/µL). Concentration of the ligand in the injection syringe was chosen to ensure 2–3-fold excess of the positive charge of the ligand over the negative charge of the DNA in the sample cell at the end of titration. The ligand was added in 62.5 µL of peptide or peptide (5 mg/mL) with DOTAP solution were added to each well; 75 µL of peptide or peptide (5 mg/mL) with DOTAP solution was diluted 5 times by HBS. HBS solution is 21 mM Hepes–NaOH buffer containing 135 mM NaCl, 5.0 mM KCl, and 0.76 mM Na2HPO4, pH 7.4. The data from the transfection experiments carried out with DOTAP are indicated as “control” in the corresponding figures presented in the results section. In transfection experiments with peptides, the stock DNA solution (concentration 1.4 mg/mL) was diluted 13 times by HBS buffer prior to addition of transfection agent. When both DOTAP and peptide were used in transfection (typically at 1:1 peptide:DOTAP molar ratio), they were mixed before addition of the DNA solution. The mixture of DNA with transfection agent(s) was incubated at room temperature for 30 min. The amount of DNA added per one 60 mm dish was typically 5.6 µg (unless otherwise specified). For a number of transfection experiments, a 5 times lower concentration of DNA in the solution was used (keeping the same amount of DNA per dish); stock DNA solution was diluted 5 times by HBS prior to addition of transfection agent. Before addition of transfection mixture, the cell growth medium was removed and cells were washed once with PBS buffer. Transfections were then performed in DMEM (5 mL/60 mm dish) without FBS. After the cells were incubated with the complexes for 5 h at 37 °C in a humidified 5% CO2 atmosphere, the complex-containing medium was removed and replaced with 5 mL of growth medium. Following an additional 48 h of incubation, the cells were collected for flow cytometric analysis.

**Flow Cytometric Analysis.** Cell transfection analysis was carried out with pEGFP-N1 plasmid complexes, following the procedure described above. After the final 48 h of incubation, cells in each well were rinsed with PBS to remove dead cells. Microscopic observation (data not shown) indicated that the dead cells lost adhesion to the well surface. Cells were harvested by trypsinization and washed once with PBS and then resuspended to a final volume of 1 mL in PBS buffer. Cells expressing green fluorescent protein were enumerated by fluorescence-activated cell sorting (FACS)Calibur (BD Biosciences, San Jose, CA) using the green channel FL-1H. A total of 3 × 104 events were counted for each sample. Data were analyzed with WinMIDI (version 2.5) software. Counts were filtered by size: events with small (cell debris) and large (cell agglomerates) sizes were discarded. Transfection efficiency is measured as a ratio of fluorescent cell count to total cell count. Then, relative transfection efficiency was determined by comparison with the value obtained in a parallel control experiment.

**Cytotoxicity Assay.** Cytotoxicity was evaluated by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cells were grown in 96-well plates (Iwaki, Tokyo, Japan) at an initial density of 1.5 × 103 cells per well in 75 µL of growth medium for 16 h at 37 °C, after which the growth medium was removed and cells were washed with PBS twice. Then 75 µL of DMEM without FBS and containing 25 µL of peptide or peptide (5 mg/mL) with DOTAP solution were added to each well; 75 µL of DMEM with 25 µL of PBS solution without peptide was applied in control wells. After incubation (37 °C, 1 h), medium and peptide were removed and 12.5 µL of a 5 mg/mL MTT stock in sterile PBS was added to each well. Following an additional incubation at 37 °C for 4 h, the MTT solution was removed and 100 µL of DMSO was added to each well to dissolve crystals. The optical density of each well was measured at 590 nm using a microplate reader (Bio-Rad, Hercules, CA) and expressed as a percentage relative to control cells.
Results

Design and Synthesis. The present work exploits a repeated branching design that uses the linear ε-oligolysine as an extended scaffold and their α-amino groups for functionalization with pendant groups of short α-peptides. Such a design has the advantages of design flexibility and synthetic expediency. The design flexibility is the ability to adjust the side-chain functionalities of the branching α-peptide motifs. The peptides contain an ε-peptide hydrophobic backbone tethered with a basic oligopeptide that can be synthesized unambiguously and honed with arbitrary net charge, hydrophobicity, and chain length. The design provides synthetic expediency by allowing a concurrent synthesis of all branched α-peptides tethered on the ε-peptide backbone during the solid-phase synthesis to save many synthetic steps.

Figure 1A illustrates the linear ε-oligolysine peptide in comparison with the normal α-oligolysine. Envisioned biophysical advantages of having this architecture include a flexible backbone derived from ε-oligolysine to facilitate DNA compaction and the pendant oligopeptide moiety at the α-amino side chain to enable optimization of delivery efficacy as well as cell targeted delivery. These peptides are also expected to be more stable in the cell as compared to a backbone based on natural α-peptides, due to the resistance of the ε-peptide bond to protease degradation. These properties, combined with the known nontoxic and safe nature of microbially produced ε-PLL, make these molecules attractive as a basis for a nonviral cationic gene delivery vehicle. We synthesized the linear ε-oligolysines of chain length varying from 5 to 10 (Figure 1A) as well as the α-amino derivatives containing either of the side chains L, Y, R, YR, and LYR (Figure 1B). For the present study, the LYR side chain motif was chosen because of the favorable membrane penetration capacity (J. P. Tam et al., unpublished observation), coupled with antimicrobial activity, of similar ε-lysine based peptides. The shorter side chains serve to test effects of amino acids with additional charge and/or of hydrophobic nature. The side chain can include histidine for improved endosomal release and/or cell targeting motifs such as RGD for specificity, while a block polymeric design would enable a flexible relative amount of such motifs to be introduced. These elements will be pursued in future work.

The peptides were prepared using Fmoc chemistry on a TFA-stable resin support as illustrated in Figure 1B. In brief, the synthesis was performed in two stages: stepwise synthesis of the ε-lysine backbone and then concurrent synthesis of all pendant α-peptides to complete the branched molecules, followed by final deprotection and the release of the peptide by a peptide bond to cleavable by HF or trifluoromethanesulfonic acid (TFMSA). Stepwise elongation by Fmoc chemistry of Boc-L-Lys(Fmoc)-OH to ε-Lys₉ and then capped by Boc-L-Lys(2-Cl-Z)-OH for protection of the final epsilon amine afforded the ε-(Boc-Lys)₁₀ backbone. In the second stage, while still attached to the resin support, all the protected Boc-groups of ε-(Boc-Lys)₁₀-ε-Boc-Lys(2-Cl-Z) were then removed under 50% TFA conditions. The unprotected α-aminos were utilized for generation of α-amino acid derivatives again by Fmoc chemistry using a “controlled polymerization” approach in which all branching α-peptides are elongated simultaneously. For the pendant tripeptide branches of ε-(LYR)K₁₀, α-amino acid derivatives Fmoc-L-Arg(Pbf)-OH, Fmoc-L-Tyr(Bu)-OH, and Fmoc-Leu-

OH were coupled consecutively by DCC/HOBt•H₂O in a mixed solution of DMF and DCM. HF cleavage or 10% TFMSA in TFA removed all protecting groups and ε-(LYR)K₁₀ from the resin support.

Biophysical Characterizations. Comparison of the DNA compaction ability of the novel ε-peptides has been performed with biophysical methods. Their effectiveness in inducing DNA compaction has been quantified by the oligopeptide charge concentration needed for a 50% increase in the static 90° light scattering intensity. Investigation of the length dependence for the linear peptides as well as a comparison of the effect of the side chain for the α-amino-substituted derivatives was conducted. For the linear ε-peptides we also compared this ability at two different concentrations of monovalent KCl salt concentrations, motivated by a recent investigation of differences in the DNA nanoparticle behavior in the presence of α-PLL and α-oligo(L-lysine) of varying length. That study detected an anomalous reversal of the salt dependence depending on the length of the peptides. Such investigation of the salt dependence is of relevance for characterization of the polyelectrolyte properties of DNA with cationic peptides as counterions. We further investigated the size of DNA nanoparticles formed by means of dynamic light scattering, and fluorescence microscopy is used to visualize the compaction of DNA. Finally, compaction is verified by isothermal titration calorimetry which gives information on the thermodynamics of cation binding to DNA. Our main objective in these biophysical experiments was the characterization of the new peptides, while some comparison with the two commercially obtained samples of ε-PLL (with the average degree of polymerization of 31) and α-PLL (degree of polymerization 40) was also included since the preliminary validation of transfection that we report is compared with those systems.

Static Light Scattering. Figure 2A shows typical curves for titration of peptides into DNA solutions. Formation of compact DNA structures is accompanied by a steep increase of scattered light. The log EC₅₀ values (the charge concentration of peptide at the midpoint of the scattering intensity curve) of the linear unsubstituted ε-oligolysines plotted against their degree of polymerization (n) are displayed in Figure 2B for experiments conducted at two different concentrations of monovalent salt (5 and 100 mM KCl). There is a decrease in the EC₅₀ values with the increase of ε-oligolysine charge, with most of the change in compaction efficiency occurring in the range n = 5–7 for both salt concentrations. The EC₅₀ value of the lowest charged ε-oligolysine, ε-K₅, is ~100-fold higher than that of ε-K₁₀ (at the same oligopeptide charge concentration). Increased degree of polymerization from 10 to 31 produces a small but noticeable decrease in EC₅₀ both at 5 and 100 mM KCl. For n > 7, compaction of DNA is more effective in 100 mM KCl than in 5 mM KCl, that is, the salt dependence is reversed compared to the smaller peptides in agreement with observations for α-(L-lysine) in comparison with α-PLL. Figure 3 compares the log EC₅₀ values of substituted ε-K₁₀ homologues with different side chains: ε-K₁₀, ε-(L)K₁₀, ε-(Y)K₁₀ (charge +10); ε-(R)K₁₀, ε-(YR)K₁₀, and ε-(LYR)K₁₀ (charge +20). The condensing efficacy is ε-(L)K₁₀ < ε-(Y)K₁₀ < ε-(R)K₁₀ < ε-(YR)K₁₀ < ε-(LYR)K₁₀. Addition of hydrophobic and aromatic amino acids to ε-K₁₀ does not increase compaction capacity of the ε-oligolysines. The charged group of the arginine side chain is also seen to be less effective in DNA compaction than the amino group in the ε-oligolysine. Only the combination of arginine, tyrosine and leucine results in more efficient compaction potential for ε-(LYR)K₁₀ com-
T2 DNA, 1 mM Tris, pH 7.3; µmM Tris, pH 7.3. Error bars represent the standard deviation from the absence of ε-compaction agent illustrated with the peptide of T2 DNA molecules in the absence and in the presence of shown). Figure 3. Influence of the side chain of oligopeptide on T2 phage DNA condensation. Concentration of T2 DNA is 4 µM; EC50 is ligand concentration expressed in µM of charged groups; 100 mM KCl, 1 mM Tris, pH 7.3. Error bars represent the standard deviation from three separate experiments.

Dynamic Light Scattering. We determined the effective hydrodynamic diameters of T4GT7 and plasmid DNA condensates formed in the presence of ε-oligolysines and α-PLL. Characterization of the hydrodynamic radius of condensates obtained by DLS is of particular relevance for systems showing good transfection ability, given the interest in the potential relation between such nanoparticle size and the in vitro transfection efficiency, a relation which is not clearly established.3

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Data on effective diameters measured at the plateau of the corresponding titration curves (similar to those obtained by SLS and shown in Figure 2A) for the T4GT7 and plasmid DNA solutions in 100 mM KCl are presented in Table 1. The effective diameters of fully compacted T4GT7 DNA are between 300 and 600 nm. Results for both T7GT7 and plasmid DNA depend on the compaction agent with the more compact particles formed by ε-(LYR)K10; linear ε-oligolysines produce larger particles (data not shown). The DLS data are consistent with results obtained by static light scattering. For the plasmid DNA, DLS measurements were carried out at two different initial concentrations of DNA, 0.2 mM (70 µg/mL) and 0.04 mM (14 µg/mL). The effective diameter of the particles formed upon

Figure 4. (A) Fluorescence image of free T2 DNA molecules in extended coil conformation. (0.5 µM (0.17 µg/mL) DNA; 0.5 µM DAPI, 1 mM Tris, pH 7.3). (B) Fluorescence image of compacted T4 DNA molecules; the condensing agent used was ε-(R)K10 at concentration 0.1 µM. (0.5 µM (0.17 µg/mL) DNA; 0.5 µM DAPI, 100 mM KCl, 1 mM Tris, pH 7.3). Typical images of DNA molecules are highlighted by ovals.

Table 1. Size (Effective Diameter) of DNA Condensates Formed in the Presence of α- and ε-oligolysines in 100 mM KCl

<table>
<thead>
<tr>
<th>condensing agent</th>
<th>size of T4GT7 DNA-condensates</th>
<th>size of plasmid DNA-condensates</th>
<th>size of plasmid DNA-condensates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε-(LYR)K10</td>
<td>374</td>
<td>188</td>
<td>97</td>
</tr>
<tr>
<td>ε-PLL</td>
<td>455</td>
<td>254</td>
<td>138</td>
</tr>
<tr>
<td>ε-K10</td>
<td>487</td>
<td>293</td>
<td>140</td>
</tr>
<tr>
<td>α-PLL</td>
<td>556</td>
<td>367</td>
<td>131</td>
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Airah provides large particles (data not shown). The DLS data are consistent with results obtained by static light scattering. For the plasmid DNA, DLS measurements were carried out at two different initial concentrations of DNA, 0.2 mM (70 µg/mL) and 0.04 mM (14 µg/mL). The effective diameter of the particles formed upon...
condensation of plasmid DNA depends on DNA concentration and is reduced when DNA concentration is decreased (Table 1). For plasmid DNA at low concentration (0.04 mM), it was possible to measure the size of uncondensed DNA molecules which was about 800 nm in agreement with estimation calculated from a flexible wormlike chain model of the DNA. We also measured the size of plasmid DNA compacted using the commercial transfection agent, DOTAP, and using DOTAP + ϵ-(LYR)K10 at concentrations mimicking conditions of the transfection experiments (see Experimental Section) and the size was 163 and 198 nm with or without added ϵ-(LYR)K10, respectively, in agreement with previous studies demonstrating decreased nanoparticle size for the polycationic/ligandophore mixture as compared to the liposome system.19,26–28 For plasmid DNA, the dependence of particle size on DNA concentration indicates that high concentration aggregates of several DNA molecules are formed.

**Isothermal Titration Calorimetry.** We measured the enthalpy of ligand–DNA interaction (ΔH) in dependence of charge ratio (positive charge of added ligand/negative charge of DNA). For comparison, values of ΔH are normalized by charge of the ligand. In Figure 5A, data determined for ϵ-oligolysines as a function of degree of polymerization is shown (rhombi). The behavior of ϵ-K10 derivatives is special (Figure 5A); the positions of the peaks are around a charge ratio 0.7–0.8 for the substituted ϵ-K10 derivatives. The behavior of the ϵ-PLL (degree of polymerization is 31) is special (Figure 5A); the substituted ϵ-PLL, the liposome formulation, we have tested the transfection enhancement effect that mixture of such peptides with DOTAP produce, as compared to peptide or DOTAP alone. This formulation is motivated by the documented finding that polycationic peptides induce potentiation of transfection for liposomes.13–15 Additionally, for those selected ϵ-peptides that exhibited significant transfection, we also performed a cell viability assay in order to establish their cytotoxic properties in comparison with the controls DOTAP and α-PLL.

**In Vitro Characterizations.** In order to compare the ability of the ϵ-peptides for in vitro transfection and test the hypothesis that these systems comprise an alternative to improved design of α-PLL peptide based delivery, we have performed a preliminary study of transfection efficacy for these new peptides in HeLa and 293F cell lines. For comparison, we also included the commercial liposome transfection agent DOTAP. In addition, we have tested the transfection enhancement effect that mixtures of such peptides with DOTAP produce, as compared to peptide or DOTAP alone. This formulation is motivated by the documented finding that polycationic peptides induce potentiation of transfection for liposomes.13–15 Additionally, for those selected ϵ-peptides that exhibited significant transfection, we also performed a cell viability assay in order to establish their cytotoxic properties in comparison with the controls DOTAP and α-PLL.

**Cell Transfection.** Figure 6 shows the transfection efficiency in HeLa (Figure 6A) and in 293F cells (Figure 6B) mediated by the different ϵ- and α-oligolysines and control transfection performed with DOTAP. Transfection was carried out using two different concentrations (but the same total amount) of plasmid DNA, 120 and 24 μg/mL. All ϵ-oligopeptides, as well as α-PLL, ϵ-PLL, the liposome DOTAP (with and without all of the peptides), were investigated but only data for systems demonstrating significant transfection are shown. In Figure 6 relative transfection efficiency is shown in logarithmic units, that is, as Due to the polydispersity of the commercially purchased α-PLL sample, which renders the interpretation of heat capacity data unreliable, data have not been obtained for this system.

The enthalpy of oligocation–DNA interaction is always positive; that is, the driving force of ligand binding is the entropic gain upon release of monovalent cations and water upon binding. A positive enthalpy is also a signature of ligand interaction with the DNA minor groove.30 Derivatives of ϵ-K10 containing leucine show more positive values of ΔH than the linear peptides; tyrosine- and arginine-containing ligands (except with leucine, LYR) display less positive enthalpy than ϵ-K5 - ϵ-K10 oligolysines. These differences in ΔH might reflect different contributions of electrostatic and hydrophobic ligand–DNA interactions (generally showing positive enthalpies in ITC measurements)29,31,32 and specific binding which usually brings favorable (negative) contribution to the binding enthalpy. In particular, higher values of ΔH of the leucine-containing oligolysines correlate with high positive values of ΔH reported in ITC studies of DNA interaction with the hydrophobic lipospermine32 and with different cationic lipids.31

All the ITC data demonstrate DNA condensation by appearance of either a peak or a sharp discontinuity, both in raw data (heat capacity versus added titrant; data not shown) and in ΔH. Significant contribution of the condensation peak to the titration curves complicates the application of simple schemes (one or two site binding models) which would allow extraction of complete thermodynamic information on peptide–DNA binding from the ITC curves. The contribution of DNA condensation to the total heat effect is higher than similar effects reported for cobalt(III) hexammine and polyamines.29 The positions of the peaks are around a charge ratio 0.7–0.8 for the substituted ϵ-K10 derivatives. The behavior of the ϵ-PLL (degree of polymerization is 31) is special (Figure 5A); the ϵ-PLL efficiently condenses DNA at the very early stages of titration with a large positive peak seen at about 0.5 charge ratio.

**Figure 5.** Results of isothermal titration calorimetry. Values of enthalpy at 298 K calculated from titration curves were normalized relative to the charge of the cationic ligand and plotted vs ratio of added ligand charge to charge of the DNA. (A) Influence of degree of polymerization of ϵ-oligolysines on enthalpy of ligand–DNA interaction. For comparison titration of the same DNA sample by solution of CO(NH2)6Cl6 is shown (rhombi). (B) Influence of side chain of ϵ-K10 derivatives on enthalpy of ligand–DNA interaction.

![Graph](image-url)
cells (Figure 6B). The linear unsubstituted
the present work showed noticeable transfection in the 293F
which show GFP transfection are displayed: (A) HeLa cells; (B)
has been used to sort the cells expressing GFP and only peptides
+ GFP in experiment with peptide (or peptide
the logarithm of the ratio of the number of cells expressing
GFP in experiment with peptide (or peptide + DOTAP) to
the same value in a positive control measurement
(when using only DOTAP as transfection agent). For the substituted
ε-oligolysine samples, the ε-(LYR)K10, can significantly
increase transfection efficiency in HeLa cells as compared to
the liposome transfection agent. Combination of either of
ε-(YR)K10 or ε-(LYR)K10 with DOTAP leads to considerably
increased transfection in the HeLa cells.13

Compared to HeLa cells, several of the peptides tested in
the present work showed noticeable transfection in the 293F
cells (Figure 6B). The linear unsubstituted ε-K10 and ε-PLL
are more effective delivery vectors than α-oligolysines, albeit
with small effect when used without DOTAP. In combination
with DOTAP, ε-lysines are relatively good transfection agents.
For the substituted ε-lysines the system composed of DOTAP
and ε-(LYR)K10 at a molar ratio of 3:2 was found to be optimal
and can mediate up to 35% of cell transfection when DNA
concentration was 24 µg/mL; other derivatives of ε-K10 are
less efficient. For all transfection agents, decrease of DNA
concentration resulted in increased GFP translation. For
ε-(LYR)K10, the dependence of transfection on the relative
amount of the DOTAP and ε-(LYR)K10 in the DNA-condensing
mixture was studied (data not shown). A 3:2 molar ratio between
DOTAP and ε-(LYR)K10 (equal to 1:1 volume ratio) displayed
the highest transfection efficiency.

Cell Viability. The in vitro cytotoxicity of the ε-oligolysines
and α-polylysines was measured for HeLa cells, and the results
for systems that showed good transfection are displayed in
Figure 7. The cell viability has been measured for the same
peptide concentrations as the in vitro transfection experiments.
In literature, such data are sometimes presented for lower
concentration of transfection agent or for conditions not
precisely relevant to the transfection protocol for which control
data on agents like polylysine and liposome (like DOTAP)
display close to 100% cell viability. Under the present conditions
(125 µg/mL), polylysine gives 40% and DOTAP 60% cell
viability. In Figure 7 it can be seen that the unsubstituted
ε-lysines exhibit lower cytotoxicity compared to both the
standard α-PLL and the liposome DOTAP. Cells incubated with
ε-PLL and ε-K10 retained greater than 70% of their metabolic
activity. The LYR-substituted side chain displays 40% cell
viability. As expected, increased peptide concentration leads to
increased cytotoxicity. Adding 625 µg/mL of the LYR-
substituted ε-peptide to the control D (DOTAP) interestingly
results in increased cell viability while in the case of adding unsubstituted ε-oligolysines, lower cell viability is observed, as
expected on the basis of the increased total cation concentration.
These results give confidence in this design based on the ε-lysine
chain, demonstrating lower cytotoxicity as compared to the
α-lysine chain. The decrease in cell viability for the LYR side
cell chain substitution is not unexpected, given the introduction of
the highly basic arginine.

Discussion
Our results generally validate the promises in designing
chemically defined, medium-length, branched ε-lysines with
pendent α-peptides as good DNA compaction agents with
potential as delivery vectors. Although the unusual and micro-
bially synthesized ε-PLL peptides have a safety record as food
additives, they are yet to be exploited as building blocks in the
design of biomolecules with unambiguous compositions. This
chimeric branched design, using a defined length of ε-oligolysines
as a flexible and hydrophobic backbone with α-peptides
as pendent functional chains to improve on their versatility is
novel. It should be emphasized that these branched peptides
are fairly large peptides and can be appropriately called
biomacromolecules. For example, ε-(LYR)K10 is equivalent in
size to an α-peptide of 63 amino acid residue. It contains 40 α- and ε-amino acid residues, 30 of which are α-amino acids as side chains and 10 as an ε-Lys10 backbone which contains 70 atoms, equivalent in length to a 23- residue α-peptide backbone. The stepwise solid-phase synthesis an all-α-peptide with 63 residues will require 63 repetitive coupling cycles. However, the solid-phase synthesis of ε-(LYR)K10 requires only 13 coupling cycles because their branched structure permits a “controlled polymerization” approach for elongating all α-amino acid side chains simultaneously. Thus, the design bodes well in analogue study for chemically defined peptides as DNA compaction and delivery vectors which greatly reduces synthetic effort and manipulations.

There are many factors which contribute to the condensation of DNA with electrostatic repulsion between highly negatively charged DNA polyions being the major force opposing formation of compact DNA structures.1,2 In the presence of multivalent cations, ion correlations induce attraction that mediates aggregation.3,4 This effect decreases with increase of monovalent salt due to increasing competition with K+ (Na+) for binding to DNA.5 Therefore, it is reasonable to expect an increase in EC50 with addition of KCl which is not the case for data of the ε-oligolysines with length n > 7, presented in Figure 2B. A similar effect was observed by Nayvelt et al. for α-PLL.25 Oligocation–DNA interaction is strongly salt-dependent46 and typically exhibits a strong dependence of binding characteristics (e.g., binding constant) versus the logarithm of monovalent salt concentration. It is unexpected that DNA compaction caused by addition of oligocationic ligands shows a rather small and often complicated salt dependence.39–41 Our data (Figure 2B) also show that direct interpretation of DNA condensation from simple polyelectrolyte theory is not straightforward. One of the reasons for this complexity might have its origin in the substantial role of the formation of bridges between the DNA molecules by lysine side chains. This contribution can be sensitive to the atomic details of compaction agent structure as well as to the structure and dynamics of oligolysine–DNA interactions.42,43

In physical characterizations, static light scattering and the ITC experiment showed that compaction of DNA occurred at an oligolysine/DNA charge ratio below 1.0 (Figure 5) which indicates that the degree of protonation of the ε-oligolysines is close to stoichiometric. For the case of ε-PLL (n = 31), DNA compaction begins at a very low degree of charge neutralization which might indicate a different mechanism of DNA condensation, perhaps dominated by bridging the DNA molecules by long chains of ε-polylysine. ε-Oligolysines also have lower pKα values than α-oligolysines. At physiological pH, the ε-oligolysines are only partially protonated and should have some lysozymotrophic effects similar to weak bases. In the case of α-lysines, the side chain amino groups (pKα = 10–11) are strongly charged at neutral pH and cannot buffer acidification of the endosome. It was postulated that polymers containing pH-sensitive groups with buffering abilities between pKα 5.0 and 7.2 could buffer the endosome and induce its collapse and the release of the endosomal content into the cytoplasm improving transfection efficacy.44 On the basis of this finding, cationic polymer and liposome vectors containing histidine (pKα = 5.0) have been designed and found to improve delivery in vitro as well as in vivo.42,45 The present design with flexible and controlled synthesis enables this function by introducing histidine in the side chain in future studies.

In many studies the relation between smaller particle and more effective transfection has been established (e.g., refs 3, 5, 7, 34, and 46), although this relation is not always valid.44 Optimal diameters vary from 50 to 200 nm. Our DLS results showed that under conditions of transfection recommended for application of DOTAP (DNA concentration about 50 μg/mL), plasmid DNA particles are around or larger than 200 nm with the substituted ε-(LYR)K10 forming the smallest particles, and this correlates with the high transfection efficiency of this compound. Decreased DNA concentration in solution before addition of the compaction agent produces smaller particles. It is likely that particles obtained at higher DNA concentration may be composed of aggregates of several plasmid molecules.

The polycationic nature of the delivery vectors can lead to cytotoxicity. Therefore interest has been directed at low molecular weight peptide delivery agents. To our knowledge, the use α-amino acid derivatives of a linear ε-oligolysine peptide is new in the context of gene delivery. The possibility of using ε-polylysine as nonviral vector has been noted.18,47,48 However, there seem to be almost no applications actually validating in vitro transfection. The only examples we are aware of is from a review article, where Smith and co-workers displayed unpublished data of one transfection experiment of the linear peptide YKA(ε-K)nWK6 and recent work on dendritic ε-poly(1-lysine)s for delivery of the DNA antisense oligomers.47 Kirby and co-workers designed, synthesized, and studied transfection capacity of a new type of amphiphilic gemini surfactant molecules with peptide head groups including the linear ε-trilysine unit.49 Dendrimeric design of α-amino-oligopeptides where the lysine side chain has one ε-linkage to the next generation in the dendrimeric hierarchy has been utilized,50 while we previously investigated dendrimeric peptides as antimicrobial agents, having a basic trilysine ε-linkage.

The substituted ε-(LYR)K10 shows superior transfection compared to ordinary α-polylysines as well as linear ε-polylysines for both cell lines investigated. In one case (HeLa) they are considerably more effective than the liposome DOTAP. Enhanced delivery to the cells is also observed when the peptides are combined with DOTAP. This interesting effect is well established and Gao and Huang13 mention several possible mechanisms, including a smaller liposome–DNA complex (as compared to the situation with liposome and DNA alone) and a protective effect of the polycation on DNA inside the liposome. In the case of the 293F cell lines, the addition of ε-(LYR)K10 does not improve transfection in combination with DOTAP. This result is in agreement with the observations that low-molecular-weight polycations comparable to the length of our peptides generally demonstrate low potentiation when added to liposome.13 The results for the HeLa cell line are interesting because generally, compared to other cell lines, transfection is low for liposomes and completely absent for standard α-peptides and the increase upon adding high molecular weight oligopeptides to liposome is moderate.13 This hints that the LYR side chain motif exhibits some cell receptor specificity in the case of HeLa cells, an issue which motivates further future studies.

Conclusions

Generally, the linear ε-lysines produce smaller particles than the corresponding α-PLL and they also exhibit better cell viability and are more effective in vitro transfection. Furthermore, design with their α-amino groups having pendent groups of short α-peptides (such as LYR) demonstrated that this basic
design can be improved for increased transfection. Cytotoxicity measurements confirmed the rationale behind our design, namely, that linear ϵ-polysynes give cell viability higher than comparable controls such as α-polysine and the liposome DOTAP. For the ϵ-polysine substituted with the basic LYR side chain, cytotoxicity is, as expected, higher but still comparable to α-polysine and in combination with DOTAP cytotoxicity is lower.

α-PLL or α-oligolysine peptide based vectors have been extensively used for DNA delivery, with interesting applications to receptor and cancer cell targeting delivery. Our preliminary results indicate that such α-oligolysine modified systems would potentially have more favorable delivery properties if they are instead based on ϵ-oligolysines. It is clear that our peptides in their present design have modest transfection capacity compared to the most promising polycationic systems in the literature. However, the point of this work has been to demonstrate that this novel design using ϵ-oligolysines can potentially improve cytotoxicity and delivery properties of existing vectors that presently are designed from α-oligolysine peptides. Therefore this study gives confidence for the approach we have initiated, and motivates further studies on improved design and with a wider range of cell lines to achieve cell targeted delivery. Such work is currently under way in our laboratory.

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Nomenclature
Boc-Lys(2-Cl-Z)-OH, N-(tert-butoxycarbonyl)-N-(2-chloro-benzoxycarbonyl)-l-lysine
Boc-Lys(Fmoc)-OH, N-(tert-butoxycarbonyl)-N-(9-fluorenylmethoxycarbonyl)-l-lysine
DAP1, 4,6-diamidino-2-phenylinole
DCC, N,N'-dicyclohexylcarbodiimide
DMF, dimethylformamide
DMEM, Dulbecco’s modified Eagle medium
DMSO, dimethyl sulfoxide
DOTAP, N-[1-(3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate
FBS, fetal bovine serum
GFP, green fluorescence protein
HOBt·H₂O, N-hydroxybenzotriazole·water
ε-K5 . . . ε-K10, oligomers of ε-polyl-lysines with degree of polymerization from 5 to 10
ε-(L)K10, ε-(Y)K10, ε(R)K10, ε(YR)K10, ε-(LYR)K10, α-substituted ε-K10 homologues, amino acids in parentheses are the substitution groups
MBHA, methylbenzydriamine
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS, phosphate-buffered saline
α-PLL, α-polyl-lysines) (degree of polymerization, 40–70)
ε-PLL: ε-polyl-lysine) (degree of polymerization, 31)
PMMA, poly(methyl methacrylate)
RP-HPLC, reversed phase high-performance liquid chromatography

References and Notes
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