Stability of Peptide-Condensed Plasmid DNA Formulations

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Abstract □ Low molecular weight homogeneous peptides were used to form peptide/DNA condensates. A peptide possessing 18 lysines was found to protect plasmid DNA from serum endonuclease and sonicative-induced degradation whereas a shorter peptide possessing 8 lysines dissociated in 0.1 M sodium chloride and failed to protect DNA from enzymatic degradation. Peptide-condensed DNA showed no change in the ratio of supercoiled to circular DNA following 100 W sonication for up to 60 s and was able to transfect HepG2 cells with equivalent efficiency as untreated condensed plasmid DNA. Alternatively, uncondensed plasmid DNA was rapidly fragmented by sonication and serum endonucleases and resulted in negligible gene expression following condensation with peptide. Cationic lipid/DNA complexes were only partially effective at stabilizing DNA in serum compared to the complete stabilization afforded by peptide/DNA condensation. These results indicate that the stabilization afforded by condensation with a peptide protects DNA during formulation and preserves its structure in serum. These functions are important to achieve optimal gene expression from a nonviral gene delivery system.

Introduction

The development of optimized plasmid DNA dosage forms for gene therapy requires an understanding of DNA stability during formulation and during transport to the target site.[1] To accomplish optimal and prolonged gene expression the delivery vehicle plays an important role in protecting DNA from physical, chemical, and enzymatic destruction. Delivery vehicles that bind to DNA and facilitate transport to cellular targets include peptides,[2] glycopeptides,[3] glycosylated polymers,[4,5] cationic lipids,[6−10] liposomes,[11,12] and PLGA microspheres.[13]

During fabrication of a delivery system the structure of DNA can be altered by shear stress to convert supercoiled plasmid to open circular, linear, or even fragmented DNA.[8] Shear stress generated by ultrasonication is a common approach used to minimize the particle size of a colloidal drug delivery system. Despite its ability to fragment DNA, several previous studies have used sonication as a means to increase the uptake of DNA by cells or to reduce particle size of colloidal DNA delivery systems.[14−16] The transfer of plasmid DNA into yeast cells in culture was reported to be improved 20-fold using mild sonication (2 W) for 30 s although further sonication led to significant DNA fragmentation and a decrease in the transfection efficiency.[14] Higher intensity sonication was reported to improve the encapsulation efficiency of DNA into liposomes, but the stability of the encapsulated plasmid DNA was only examined by low resolution gel permeation chromatography.[15] A cationic lipid/DNA formulation was also recently reported to be stable to 50 W sonication for 60 s to reduce particle size heterogeneity.[16]

Likewise, serum endonucleases can act in vivo during transport to fragment unprotected DNA and thereby abolish its ability to transform cells. Only a few studies have examined the role of the drug delivery vehicle in stabilizing DNA formulations in serum.[1,6,17] Uncomplexed plasmid DNA was found to be rapidly fragmented within 5 min in vivo and within 30 min during in vitro incubation in mouse serum due to the action of endogenous endonucleases.[1,17] Certain cationic lipid/DNA complexes were reported to partially protect DNA against purified DNAse I and against DNAase in human serum despite reports that cationic lipid gene transfer is greatly reduced in the presence of serum.[6,18]

Gel electrophoresis is still the most direct and sensitive approach to examine the stability of plasmid DNA in a gene delivery formulation or in serum. However, peptide and lipid delivery vehicles remain bound to DNA and interfere with its migration thereby necessitating phenol/chloroform extraction of DNA prior to electrophoresis. Other methods of analyzing DNA stability, such as gel permeation chromatography or the transformation of *Escherichia coli* or mammalian cells with extracted DNA, are less definitive compared to the direct analysis of DNA stability by gel electrophoresis since subtle alterations in DNA structure may escape detection.[14−16]

The stability profile of DNA formulations containing peptides and glycopeptides has not been reported previously. Partly, this is because of the difficulty of recovering DNA from tightly bound peptides which inhibit migration on gel electrophoresis. In the present study we have examined the influence of plasmid structure in relationship to gene expression in a peptide-mediated gene delivery system. The results not only establish important methodology that allow direct analysis of DNA stability by gel electrophoresis, but also report that peptide/DNA condensates are stable to sonicative shear stress and direct attack from serum endonucleases. These properties help to explain how peptide/DNA condensates are able to transform cells in culture in the presence of serum and suggest that a similar stability will be realized in vivo.

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Materials and Methods

CWK_8 (Alkylated Cys-Trp-Lys_8), CWK_{18} (Alkylated Cys-Trp-Lys_{18}), and dimeric CWK_{18} (K_{18}WC-CWK_{18}) were synthesized and characterized as described previously.[19] pCMVL was produced in *E. coli* and purified using a Qiagen Ultrapure-100 kit (Santa Clarita, CA). TPCK-treated trypsin was obtained from Worthington Biochemicals (Freehold, NJ). Micro BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). MEM, bovine calf serum, electrophoresis grade agarose, and LipofectAce (1:2.5 w/w dimethyldioctadecyammonium bromide and dioleolylphosphatidylethanolamine) were obtained from Gibco BRL (Gaithersburg, MD). Nru I restriction enzyme was purchased from Boehringer Mannhein (Indianapolis, IN).

Generation of Linear and Circular pCMVL—Supercoiled plasmid pCMVL[19] was linearized with Nru I which recognizes the sequence TCG/CGA at bp 206 of the pRC/CMV cloning vector from Invitrogen (San Diego, CA). One hundred units of Nru I was used to cleave 100 μg of pCMVL in 200 μL of SuRE/Cut buffer at 37 °C for 1 h. The linear DNA was purified by precipitation with 150 μL of ethanol at −20 °C followed by centrifugation at 13000 g for 5 min at 4 °C and then analyzed by 1% agarose gel electrophoresis.

Open circular pCMVL was prepared by creating single stranded nicks in the supercoiled pCMVL. The DNA (100 μg) was heated to 70 °C in 100 μL of TAE buffer, pH 8.0, for 1 h and then purified using ethanol precipitation and analyzed by gel electrophoresis.

Preparation and Characterization of Peptide/DNA Condensates—Peptide/DNA condensates were prepared at a DNA concentration of 50 μg/mL in 5 mM Hepes pH 7.4 using a stoichiometry of 0.3 nmol of peptide per μg of DNA. DNA (150 μL of 0.1 μg/μL) was added dropwise to a microfuge tube containing 4.5 nmol of peptide in 150 μL of buffer. Peptide/DNA condensates formed instantly, although physical measurements were carried out after 30 min to allow the particle size to stabilize.[19] Particle size analysis was performed on 350 μL of the undiluted DNA/peptide complex using a Nicomp 370 Autodilute Particle Sizer (Nicomp, Santa Barbara, CA).

Sonication of DNA Condensates—A 100 W Microson XL-2000 ultrasonic probe homogenizer (Kontes, Vineland, NJ) with a vibrational amplitude of 5 was used throughout the study. The probe tip was placed at $^3/_4$ depth into a 1.5 mL microfuge tube containing 300 μL of sample. The sonication time was varied from 15 to 60 s, and the probe tip was washed between samples using deionized water.

Sonicated DNA samples were analyzed by gel electrophoresis on a 1% agarose gel prepared in TAE buffer (pH 8.0, 40 mM Tris acetate, 2 mM EDTA) containing 0.5 mg/mL ethidium bromide. Prior to electrophoresis, peptide/DNA condensates (2.5 μg DNA) were digested for 30 min with 5 μg of trypsin (0.2 units) prepared in a final volume of 50 μL of 5 mM Hepes pH 7.4 in order to release plasmid DNA from the condensate. The DNA (0.75 μg/15 μL) was combined with 3 μL of loading buffer (0.25 wt % bromophenol blue, 0.25 wt % xylene cyanol FF, and 30 wt % glycerol in water) and then loaded onto the gel and electrophoresed for 1.5 h at 70 V. DNA bands were visualized following destaining of the ethidium bromide on a transilluminator and photographed on Polaroid 667 black and white film.

DNA condensates were prepared using either CWK_8, CWK_{18} or dimeric-CWK_{18} and then adjusted to 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1 M sodium chloride and sonicated for 60 s to fragment uncondensed DNA. The samples were then digested with trypsin and electrophoresed on an agarose gel.

Serum Stability of Peptide/DNA Condensates—The DNAse activity in freshly prepared mouse serum was determined to be 4.2 units/μL according to the method of Kunitz.[20] CWK_{18}/DNA condensates (5 μg/100 μL) prepared in 5 mM Hepes pH 7.4 and 150 mM sodium chloride were combined with 100 μL of mouse serum and allowed to incubate at 37 °C for 3 h, while rapidly freezing 10 μL aliquots at intermediate time points. Prior to electrophoresis, 10 μL of 10 mg/mL SDS was added to each aliquot along with 5 μL of 68 mM EDTA and 3 μL of loading buffer, and samples were applied to an agarose gel containing 0.05 w/v% SDS and electrophoresed for 1 h at 70 V. The gel was destained in water for 24 h to remove SDS and increase the detection of the bands by ethidium bromide.

The stability of a LipofectAce/DNA complex was also studied by combining 30 μL of LipofectAce with 20 μL of 5 mM Hepes containing 150 mM sodium chloride and 50 μL of DNA (10 μg/100 μL of 5 mM Hepes pH 7.4). The complexes were then combined with 100 μL of mouse serum, incubated at 37 °C for 3 h while removing time points, and then electrophoresed as described above for peptide/DNA condensates.

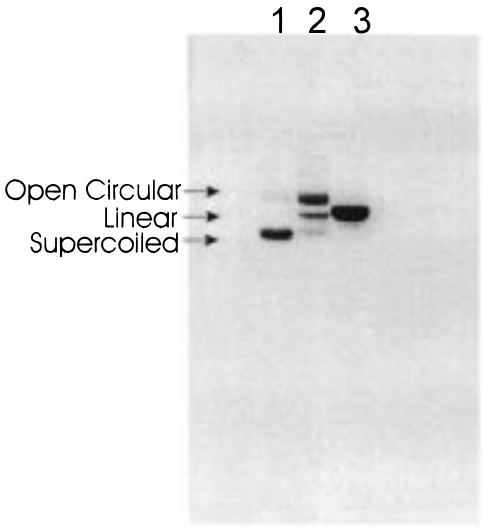

Figure 1—Gel electrophoresis of supercoiled, circular, and linear DNA. The result of converting supercoiled pCMVL (lane 1) to open circular (lane 2) and linear (lane 3) DNA is shown. Each lane was loaded with 0.5 μg of DNA and electrophoresed as described in Materials and Methods. The result of gene transfer with each type of DNA is described in Figure 2.

In Vitro Gene Transfer—Gene transfer experiments were performed on HepG2 cells grown to 40% confluence in MEM supplemented with 10% fetal bovine serum as reported previously.[19] The DNA condensate (10 μg of DNA) was applied to the cells in 2% fetal bovine serum with 80 μM chloroquine and allowed to incubate for 5 h after which time the media was replaced with MEM containing 10% fetal bovine serum.[19] After 24 h, cells were harvested and analyzed for the presence of luciferase. The expression level of luciferase was normalized for protein in each well, and the relative light units were converted to fmol of luciferase/mg of protein using a standard curve as reported previously.[19] Each experimental result represents the mean and standard deviation derived from a triplicate set of transfections.

Results

To establish a relationship between plasmid DNA structure and gene transfer efficiency, we first prepared circular DNA by base hydrolysis of supercoiled DNA. In its native form, plasmid DNA exists as a mixture of both supercoiled and open circular DNA forms that resolve on gel electrophoresis (Figure 1, lane 1). Treatment of plasmid DNA at pH 8.0 with elevated temperature (70 °C) accelerated the hydrolysis of supercoiled DNA to form predominantly circular DNA within 2 h (Figure 1A, lane 2). Linear DNA was prepared by restriction digestion with Nru I, which cleaved the plasmid prior to the CMV promoter, leaving the essential coding region for luciferase and the CMV promoter intact (Figure 1, lane 3).

Condensation of DNA with peptides possessing lysine repeats of either 18 or 36 residues resulted in the formation of fully condensed 50−70 nm diameter particles.[19] Linear, supercoiled, and circular DNA condensates could not be distinguished through particle size on QELS analysis (Table 1). However, their transfection efficiency was significantly different (Figure 2). The gene transfer efficiency of circular DNA was only reduced 10% compared to supercoiled DNA whereas linear DNA condensates were nearly 90% less efficient at transfecting cells and uncondensed DNA was inactive in mediating expression.

We then analyzed the influence of shear stress and endonuclease attack on the stability of supercoiled plasmid

Table 1—Particle Size Analysis of Peptide/DNA Condensates[a]

DNA morphology	particle size population	
	diameter[b](nm)	σ[c] (nm)
supercoiled	46.9	32.4
circular	61.0	36.0
linear	72.6	37.4
sonicated[d]	44.8	28.8

[a] Peptide/DNA condensates were prepared at a concentration of 50 μg/mL of DNA and at stoichiometry of 0.3 nmol of CWK_{18} per μg of DNA. [b] Represents the mean diameter of particles. [c] Standard deviation of the population. [d] DNA sonicated for 60 s prior to condensation with CWK_{18}.

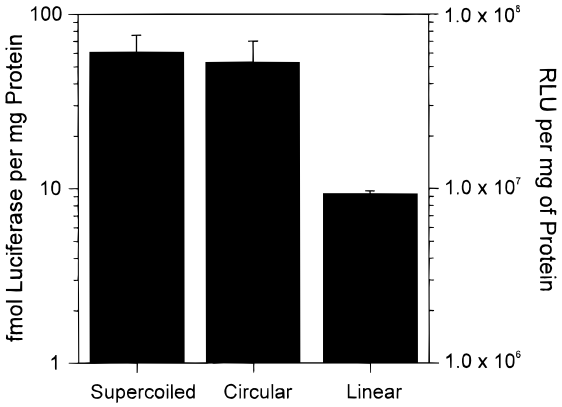

Figure 2—Gene transfer efficiency of supercoiled, circular, and linear DNA. The transfection efficiency of the three types of DNA shown in Figure 1 were compared. Each transfection utilized 10 μg of DNA combined with 3 nmol of CWK_{18} in 200 μL of 5 mM Hepes pH 7.4. The results establish a slight (10%) reduction in gene transfer when using open circular DNA and a 90% reduction when the DNA is linear relative to supercoiled DNA. Identical transfections with uncondensed DNA only resulted in 0.001 fmol of luceriferase per mg of protein.

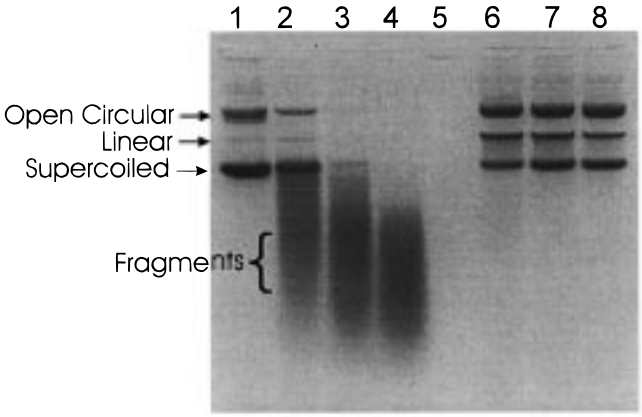

Figure 3—Stability of peptide/DNA condensates to ultrasonication. Gel electrophoresis with ethidium staining was used to demonstrate the fragmentation of plasmid DNA on sonication for 15, 30, and 60 s (lanes 2, 3, and 4) relative to standard DNA (lane 1). After complexation with CWK_{18} and sonication for 60 s, plasmid DNA failed to migrate on gel electrophoresis (lane 5). Treatment of the sonicated condensed DNA with trypsin restored migration but led to the formation of some linear DNA (lane 6). Omission of sonication (lane 7) or both sonication and condensation with CWK_{18} (lane 8) resulted in identical banding patterns indicating that linear DNA was an artifact of contaminated trypsin.

DNA. Condensed and uncondensed DNA were challenged with 100 W sonication for up to 60 s. Sonication of uncondensed plasmid DNA for as little as 15 s resulted in extensive fragmentation (Figure 3, lane 2), while prolonged sonication for 30−60 s further fragments DNA to form a finite size distribution of oligonucleotides as previously reported (Figure 3, lanes 3 and 4).[21]

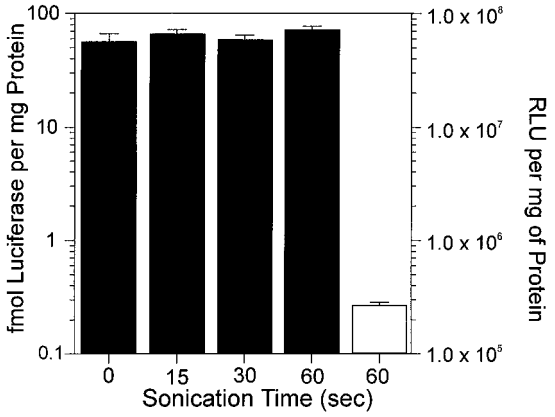

Figure 4—Gene transfer efficiency of sonicated peptide/DNA condensates. The expression of luciferase following in vitro transfection of HepG2 cells is compared for CWK_{18}/DNA condensates subjected to 0, 15, 30, and 60 s of sonication, relative to plasmid DNA sonicated for 60 s and then condensed with CWK_{18} (open bar).

In contrast, electrophoretic analysis of peptide condensed DNA sonicated for 60 s resulted in an empty lane (Figure 3, lane 5). This results from the failure of peptide/DNA condensates to dissociate and to stain with ethidium bromide. Trypsin was used to hydrolyze CWK_{18}, allowing the plasmid DNA to migrate and stain normally in the gel (Figure 3, lane 6). The formation of 10% linear DNA was the result of endonuclease contamination of trypsin. This was deduced from control experiments in which trypsin digestion of peptide/DNA condensates as well as uncondensed plasmid DNA both produced a linear DNA band (Figure 3, lane 7 and 8).

Peptide/DNA condensates were sonicated for up to 60 s and used to transfect HepG2 cells to establish that sonication also does not alter gene transfer efficiency. The results showed no change in gene transfer efficiency for sonicated DNA relative to unsonicated condensates (Figure 4). Fragmented DNA also formed small (45 nm) peptide/DNA condensates (Table 1), but these showed negligible gene expression activity (Figure 4).

Since the stability of peptide/DNA condensates may also be influenced by the solution ionic strength, we analyzed the dissociation of peptide/DNA condensates in the presence of increasing sodium chloride concentration in an attempt to disrupt the peptide/DNA binding. However, the failure to detect any DNA bands by gel electrophoresis after incubating the condensates with up to 5 M sodium chloride suggested that either the condensates failed to dissociate or they reformed during gel electrophoresis.

To distinguish between these alternatives, peptide/DNA condensates were treated with sodium chloride at concentrations ranging from 0 to 1 M and then sonicated for 60 s to fragment any uncondensed DNA. Trypsin was then added to hydrolyze CWK_{18} and allow the DNA to migrate into the gel during electrophoresis. The resulting gel established that peptide/DNA condensates dissociate at sodium chloride concentrations below 1 M as revealed by the presence of fragmented DNA (Figure 5).

The validity of the method was established by comparing the sodium chloride concentration required to dissociate three different peptides. Previously, we established that CWK_8 binds weakly to DNA but was able to fully condense DNA at a stoichiometry of 0.8 nmol per μg of DNA.[19] As observed in Figure 5A, the DNA remains protected by CWK_8 in 0.1 M sodium chloride (lane 2) but was degraded by sonication at all higher sodium chloride concentrations due to dissociation of the peptide/DNA complex. CWK_{18} possesses a significantly greater affinity for DNA resulting in complete condensation at 0.3 nmol per μg of DNA.[18] The

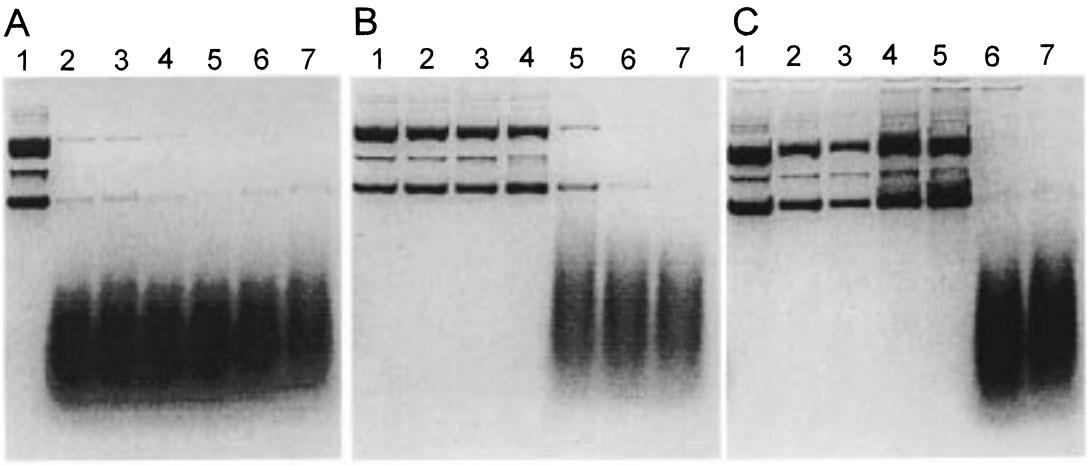

Figure 5—Sodium chloride induced dissociation of peptide/DNA condensates. The stability of peptide/DNA condensates to sonication in the presence of 0–1 M sodium chloride is demonstrated using gel electrophoresis. CWK_8 (panel A), CWK_{18} (panel B), and dimeric-CWK_{18} (panel C) DNA condensates were treated with 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1 M sodium chloride prior to 60 s sonication and trypsin digestion. The dissociation of the peptide/DNA condensate was observed at above 0.1 M for CWK_8 (panel A), above 0.4 M CWK_{18} (panel B), and above 0.6 M for dimeric-CWK_{18} (panel C) DNA condensates.

results shown in Figure 5B support this by demonstrating protection of the DNA by CWK_{18} at concentrations up to 0.4 M sodium chloride. Likewise, dimeric-CWK_{18} was previously found to have a slightly higher affinity for DNA but produced condensates that were the same size as CWK_{18}.[19] In agreement with these results, dimeric-CWK_{18}/DNA condensates were more stable as demonstrated by their ability to resist sonicative-induced fragmentation up to 0.6 M sodium chloride.

To study the serum stability of peptide/DNA condensates required modification of the gel electrophoresis approach. In the presence of serum, trypsin was incapable of completely hydrolyzing the peptide to release DNA, resulting in bands that migrated slower than open circular DNA on gel electrophoresis (data not shown). Consequently, SDS was found to be an effective agent to dissociate the peptide/DNA complexes present in serum. The optimal result was obtained by adding 0.05 w/v% of SDS in the agarose gel and running buffer along with 0.3 w/v% SDS in the loading lane.

Incubation of either DNA, peptide/DNA condensates, or LipofectAce/DNA complexes with freshly prepared mouse serum adjusted to 0.15 M sodium chloride was used to establish the in vitro stability of DNA. Direct analysis of the incubation time points by SDS-agarose electrophoresis established that uncondensed DNA rapidly converted from supercoiled to circular DNA and then began to form linear DNA within a 5 min incubation period (Figure 6A, lane 2). Further incubation resulted in progressive formation of linear DNA which then degraded to smaller oligonucleotides within 1 h and resulted in complete fragmentation of DNA within 3 h (Figure 6A, lanes 6−8). Conversely, CWK_{18} condensed DNA was stable when exposed to serum during a 3 h incubation period (Figure 6B), demonstrating the complete preservation of supercoiled DNA.

This result suggested that the dissociation of CWK_8/DNA condensates in normal saline (0.15 M) could leave DNA exposed to serum endonucleases. Incubation of CWK_8/DNA condensates with serum resulted in a degradation profile that was identical to that observed for uncondensed DNA (Figure 6C). This adequately explains the three-orders of magnitude difference in the transfection efficiency previously observed when transfecting cells with CWK_{18} and CWK_8/DNA condensates in the presence of fetal calf serum.[19]

This same rational may also explain the ineffectiveness of LipofectAce/DNA transfection in the presence of serum. To establish this point, LipofectAce/DNA complexes were incubated in serum, and time points ranging from 0 to 3 h were analyzed by gel electrophoresis (Figure 6D). Although some protective effect was conferred by the presence of the cationic lipid formulation, the DNA still underwent a complete conversion from supercoiled to circular and linear DNA within 1 h and was 50% depolymerized during the 3 h incubation.

Discussion

The stability of a DNA formulation in serum is fundamental to its successful application in vivo since premature metabolism results in the generation of fragmented DNA which lacks gene transfer potency. The utility of gel electrophoresis to study the stability of plasmid DNA complexed with peptides or lipids in a gene delivery formulation has been limited because of the interference imparted by the these biopolymers which retard or distort the electrophoretic migration of DNA. The present results establish that the addition of SDS to agarose gel electrophoresis or, the use of trypsin to remove peptide, allow direct analysis of the status of DNA in a complex formulation and in serum. The use of radiolabeled DNA will further advance these studies by allowing a quantitative analysis of DNA recovery.

In an effort to understand how the serum stability of peptide/DNA condensates correlates with gene expression, we first analyzed how changes in plasmid structure influence gene transfer efficiency. The results presented in Figures 1 and 2 demonstrate that the conversion of DNA from supercoiled to circular only has a minor effect on the gene transfer efficiency. However, subsequent conversion to linear DNA reduces gene expression by 90% whereas transformation to oligonucleotide fragments via sonication reduced gene expression nearly 1000-fold (Figure 2).

We were intrigued to find that high-intensity sonication of CWK_{18}/DNA condensates for up to 60 s failed to degrade the DNA (Figure 3). It is also interesting that sonicatively fragmented DNA was still able to bind to CWK_{18} and produce condensates that were indistinguishable in size relative to those prepared from supercoiled, circular, or linear DNA (Table 1). Thus, it is evident that the poor transfection performance of fragmented DNA is not because of the failure to form condensate but rather directly related to the stability of the DNA.

We cannot explain the mechanism by which DNA folds to produce particles of a defined size, but it must be

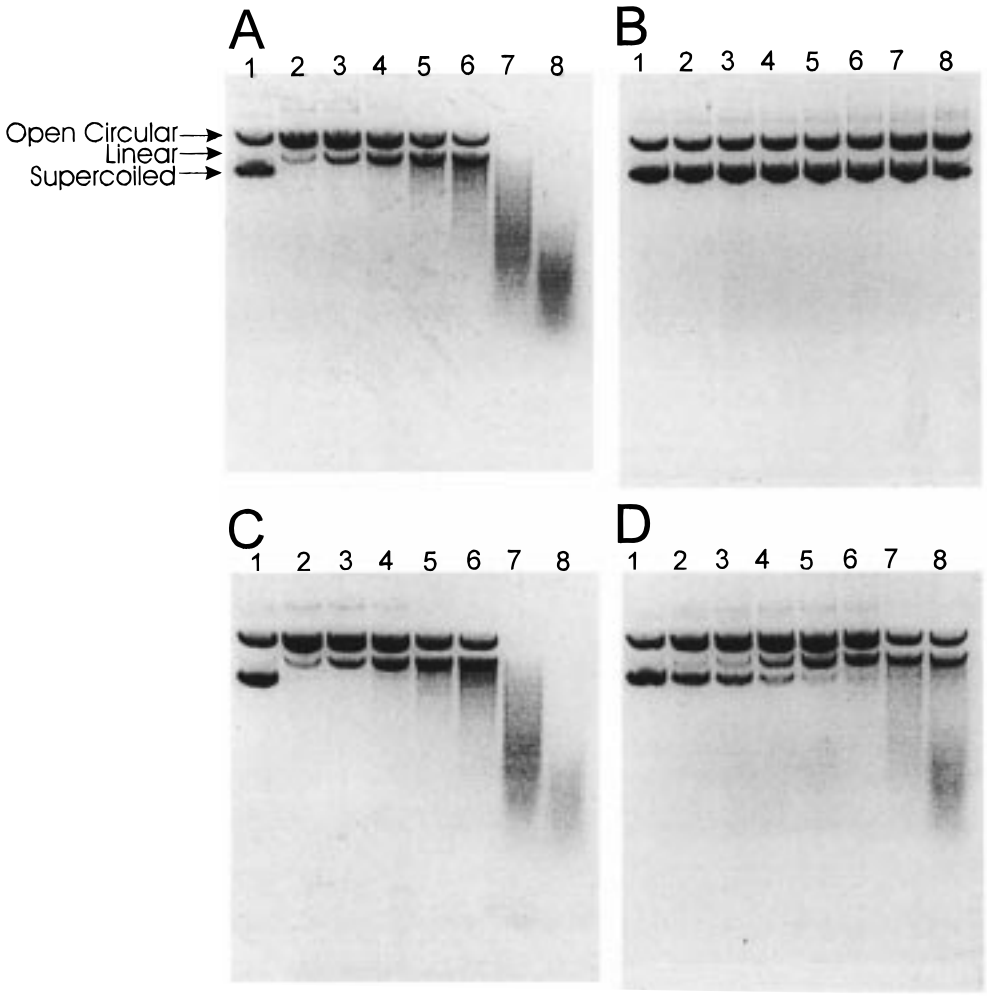

Figure 6—Serum stability of peptide/DNA condensates. Uncondensed plasmid DNA (panel A), CWK_{18}/DNA condensates (panel B), CWK_8/DNA condensates (panel C), and LipofectAce/DNA complexes (panel D) were incubated with mouse serum at 37 °C and analyzed by gel electrophoresis as described in Materials and Methods. Time points were analyzed at 0, 5, 15, 30, 45, 60, 120, and 180 min in lanes 1–8, respectively. The rapid conversion of supercoiled DNA to linear DNA is evident in panels A and C, and the formation of fragmented DNA is nearly complete at 2 h indicating a lack of protection afforded by CWK_8. LipofectAce/DNA complex showed a similar profile but the DNA fragmentation was slower than for uncondensed DNA. Panel B illustrates the protective effects of CWK_{18} during the same 3 h incubation.

intrinsically related to the nature of peptide rather than the size or polydispersity of the DNA since it is clear that peptide size influences the properties of condensates.[19,22] A practical outcome of this analysis of peptide/DNA stability is that high power sonication could be used as a means to reduce particle size while still preserving DNA structure when developing complex formulations for gene therapy that require shear stress, such as liposomes[18] or aerosols.[8]

The dissociation of peptide/DNA complexes in sodium chloride allowed for the development of an assay to define the condensation status of DNA in a complex solution. The data presented in Figure 5 clearly demonstrate that the dissociation of peptide from DNA at a defined salt concentration depends on the affinity of different peptides binding to DNA. The data not only established that CWK_{18} retains its ability to bind to DNA in 0.4 M sodium chloride but also revealed that CWK_8 dissociates at a concentration of 0.1 M sodium chloride. These results predicted that CWK_{18} would protect DNA from endonucleases in normal saline (0.15 M sodium chloride) whereas CWK_8 would not protect DNA. This proved to be the case, in that there was no distinction between DNA serum stability in the presence or absence of CWK_8 whereas the complete protection of DNA was observed when complexed with CWK_{18} (Figure 6).

An examination of the serum stability of LipofectAce/DNA complexes determined that the DNA was more stable than uncondensed plasmid DNA but still degraded to oligonucleotides within a couple of hours. These results adequately explain the lack of potency when transforming cells with uncondensed DNA and when using LipofectAce in the presence of serum. Moreover, the data suggest a possible explanation for the lack of correlation between in vitro and in vivo gene transfer results for certain gene delivery vehicles.

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