

Sustained release of plasmid DNA using lipid microtubules and agarose hydrogel

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Abstract

Non-viral gene therapy typically results in low transfection efficiencies and transient gene expression. To address these limitations, two sustained delivery systems capable of releasing functional, compacted DNA for over 50 days were designed. A luciferase plasmid was compacted with a polylysine–polyethylene glycol conjugate and released from agarose hydrogel and lipid microtubule–hydrogel delivery systems for over 50 days. The released DNA was characterized structurally using sedimentation, electron microscopy, and serum stability, and functionally using *in vitro* transfections. The released DNA retained its physical compaction and nuclease resistance and was converted from supercoiled to nicked and linear forms. Released compacted DNA produced significant gene expression *in vitro*, although at lower levels than freshly compacted DNA. Thus, hydrogels and lipid microtubules successfully provided the slow release of bioactive, compacted DNA.

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1. Introduction

Sustained gene delivery has been considered for a variety of *in vivo* applications, including wound healing [1,2], bone regeneration [3,4], and vaccination [5–8]. Materials used for the slow release of plasmid DNA include poly(DL-lactide-co-glycolide) (PLGA) [1,5–8], gels of polymethacrylic acid and

polyethylene glycol [9], sodium alginate [10], poly(ethylene-co-vinyl acetate) [11,12], gelatin [13], and collagen [2–4]. To encapsulate DNA, many polymeric delivery systems require organic solvents to dissolve the polymer and harsh physical forces to mix the immiscible water and solvent phases. Residual solvents could detrimentally affect the *in vivo* response, and forces caused by techniques such as sonication can degrade DNA [6,14]. In addition, low pH, which can occur as PLGA degrades [15], can convert supercoiled plasmids to nicked forms and can sometimes degrade DNA [6,8]. While attempts to address these concerns are underway [8,16], we

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propose to circumvent these issues using a novel method for the sustained release of plasmid DNA.

The aim of this study was to develop and characterize delivery systems capable of releasing plasmid DNA that would obviate exposure to organic solvents and harsh physical forces. Two delivery systems for the slow release of compacted DNA have been developed: agarose hydrogel (Gel), and a two-component system consisting of lipid microtubules embedded in agarose hydrogel (MT–Gel). Agarose, a natural polysaccharide derived from red algae, forms a thermoreversible hydrogel. It has been used for drug delivery applications [17,18], and no adverse inflammatory or immunological consequences have resulted from the use of agarose in vivo [19,20]. Lipid microtubules are a non-inflammatory delivery system [21] for proteins and growth factors in vitro [21–23]. These systems are injectable and can therefore be localized to specific areas in vivo. The advantages of such delivery systems include a lack of residual organic solvents, no need to mix immiscible phases, and two available formulations that provide different DNA release profiles. In addition, compacted plasmid DNA is used; compacted DNA has resulted in significant gene expression in vivo following intrapulmonary [24,25] and intradermal (unpublished data) injections, and may further facilitate non-viral gene transfection compared to uncompact, naked plasmids. This formulation of compacted DNA does not aggregate in physiological saline solution and is stable for prolonged periods of time at 4 °C [24].

2. Materials and methods

2.1. Compaction of plasmid DNA

A cationic peptide consisting of an N-terminal cysteine and 30 lysine residues (CK₃₀) was prepared by solid phase synthesis with trifluoroacetate (TFA) as the negative counterion. Polyethylene glycol (PEG, 10 kDa, Shearwater Polymers, Huntsville, AL, USA) functionalized with a maleimide group was covalently coupled to the cysteine residue of the peptide. The final conjugate, CK₃₀PEG10k/TFA, was purified by gel filtration and lyophilized.

The reporter plasmid used in this study,

pKCERlucSV5.9, contained the luciferase gene transcriptionally controlled by the elongation factor-1 α promoter and the cytomegalovirus enhancer. DNA was compacted by adding 0.9 ml of plasmid (0.22 mg/ml) in 0.1 ml aliquots to 0.1 ml of CK₃₀PEG10k/TFA (7.1 mg/ml) in sterile, deionized water while mixing vigorously. After compaction, the DNA was filtered through a 0.2- μ m syringe filter to sterilize and then concentrated 20-fold in centrifugal concentrators (Millipore, Bedford, MA, USA). After the centrifugation, 4 ml of 150 mM NaCl solution were added to the compacted DNA, and the solution was again concentrated. This changed the solvent from water to 150 mM NaCl solution without altering the compacted DNA molecules. The resulting DNA is referred to as freshly compacted DNA.

2.2. Gel delivery system fabrication

Agarose hydrogel was prepared by dissolving SeaPlaque agarose (Cambrex, East Rutherford, NJ, USA) in 150 mM NaCl for an agarose concentration of 0.75% (w/v). To prepare the samples for the Gel delivery system, 200 μ l of compacted DNA (0.625 mg/ml) were mixed with 400 μ l of 0.75% agarose at 37 °C, and triplicate samples were gelled in glass vials at 4 °C for 30 min. Negative controls contained no DNA.

2.3. MT–Gel delivery system fabrication

For the MT–Gel delivery system, lipid microtubules were fabricated from the lipid, 1,2-bis-(tricoso-10,12-diynoyl)-sn-glycero-3-phosphocholine (DC_{8,9}PC, Avanti Polar Lipids, Alabaster, AL, USA), as previously described [21]. Briefly, the lipid was dissolved at a concentration of 1 mg/ml in 70% ethanol at 55 °C. The solution was cooled to 25 °C, heated to 33 °C, and cooled to 25 °C using a refrigerated water bath (Thermo NESLAB, Portsmouth, NH, USA). Trehalose (50 mM, Sigma, St. Louis MO, USA) was added, the tubules were centrifuged (1500 \times g, 5 min), and the pellet was dried on a rotary evaporator. DNA was incorporated by rehydrating the microtubules with compacted DNA (3 mg/ml DNA in 150 mM NaCl) at a ratio of 40 μ l DNA solution to 1 mg lipid. Excess DNA was

removed by diluting the microtubules with 1 ml of 150 mM NaCl solution per mg of lipid, centrifuging (380×g, 10 min), and aspirating the supernatant.

The quantity of DNA in the microtubules was estimated as the total internal volume of the microtubule sample multiplied by the concentration of the DNA loading solution. The internal volume was based on an average diameter of 0.5 μm [26], the microtubule yield (number of microtubules per mg of lipid), and the average microtubule length. Yield and length were measured using a Nikon Eclipse TE300 light microscope (Nikon, Tokyo, Japan) and a MagnaFire digital camera (Optronics, Goleta, CA, USA) connected to a computer running Image Pro Express software (Media Cybernetics, Des Moines, IA, USA).

To prepare MT–Gel samples, 5 mg of microtubules containing compacted DNA were suspended in 200 μl of 150 mM NaCl and mixed with 400 μl of 0.75% SeaPlaque agarose solution at 37 °C. Microtubules containing 150 mM NaCl solution, without DNA, served as a negative control. Triplicate samples of MT–Gel solutions were gelled in glass vials at 4 °C for 30 min.

2.4. Characterization of sustained release of compacted DNA

Immediately after gelation of the Gel and MT–Gel systems, 250 μl of 150 mM NaCl were added to each 600-μl gelled sample, and the samples were incubated at 37 °C. Periodically, the supernatant was removed for testing and replaced with fresh solution. The DNA concentration in the supernatant was quantified by UV-absorbance at 260 nm.

Released DNA samples were evaluated using a sedimentation assay, transmission electron microscopy (TEM) visualization, serum stability assay, and in vitro transfections. In all characterization assays, freshly compacted DNA was the positive control.

2.5. Structural characterization of released DNA

A sedimentation assay was used to assess the colloidal stability of the compacted DNA. The UV-absorbance at 260 nm was quantified for freshly

compacted DNA samples (15–20 μg/ml) and released DNA samples (2–85 μg/ml) before and after centrifugation (2800×g, 1 min). A lack of sedimentation, specified as a decrease in UV-absorbance of less than 20% after centrifugation, indicates that the DNA particles are non-aggregated [27].

The size and morphology of freshly compacted and released DNA particles were examined under TEM. Freshly compacted and released DNA samples (10 μl, 20–80 μg/ml) were applied to carbon-coated copper grids (400 mesh, Ted Pella, Redding, CA, USA) for 2 min. The grids were inverted on water for 1 min, negatively stained by immersion in uranyl acetate (0.4 mg/ml in methanol) for 2 min, dipped in 100% ethanol, and air-dried. They were observed at 80 kV on a JEM 1200 EX electron microscope (JEOL, Peabody, MA, USA). The 87.5 Å spacing of catalase crystals was used to calibrate the microscope.

2.6. Serum stability assay

Nuclease resistance was evaluated using a serum stability assay and electrophoresis. Freshly compacted and released DNA samples were incubated with 75% mouse serum (Sigma) for 2 h at 37 °C. The reaction was arrested by adding ethylenediamine-tetra-acetic acid (EDTA) for a final concentration of 0.1 M. To uncompact the DNA, trypsin (12.5 mg/ml, Sigma) was added for 40 min at room temperature. Control samples included freshly compacted and released DNA that were either trypsin-digested without the serum incubation or untreated. The DNA was extracted using phenol–chloroform–isoamyl alcohol (25:24:1 volume ratio) and loaded onto a gel (0.8% agarose with ethidium bromide in Tris–borate–EDTA buffer). Fluorescent labeling of DNA decreases significantly after compaction, so 1500 ng of untreated freshly compacted DNA were added per well to enhance the ethidium bromide signal. For freshly compacted samples that were either trypsin-digested or serum-incubated plus trypsin-digested, 250 ng DNA were added per well. The mass of released DNA added per lane was dependent upon the concentration of the released DNA and ranged from 20 to 480 ng. Gels were run at 80 V for 2 h and photographed under UV-light.

2.7. Functional characterization of released DNA

The functional potency of the released DNA was tested by transfecting bovine aortic smooth muscle cells (BASMC) with DNA samples released from the delivery systems. BASMC were isolated and cultured as previously described [28]. Cells (passages 5–15) were plated in 48-well plates (12,000 cells/well) with 500 μ l of regular growth medium, consisting of Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and grown in humidified incubators at 5% CO₂ and 37 °C.

When the BASMC reached 40–60% confluency, the growth medium was removed and replaced with 500 μ l reduced-serum medium (OptiMEM, Invitrogen). Triplicate wells received 1 μ g of DNA released from the Gel or MT–Gel system. Negative control wells contained no DNA. Freshly compacted DNA and naked DNA (1 μ g per well) were also evaluated. After 4 h, the medium was removed, 500 μ l of regular growth medium were added per well, and the cells were cultured for 20 h.

Cells were lysed 24 h post-transfection by removing the growth medium, washing the wells twice with magnesium- and calcium-free phosphate buffered saline, and adding 50 μ l of Cell Culture Lysis Reagent (Promega, Madison, WI, USA) per well. After shaking for 20 min at room temperature, the cell lysate was transferred to tubes and centrifuged (7800 \times g, 5 min).

Luciferase activity was quantified by mixing 20 μ l of lysate with 100 μ l of Luciferase Assay Reagent (Promega) and measuring light emission for 10 s on an ILA911 luminometer (Tropix, Bedford, MA, USA). Total cellular protein was determined using the DC Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Luciferase activity was normalized by reporting the relative light units per microgram of cellular protein (RLU/ μ g protein).

To determine the effect of serum on the transfection efficiency, transfections were also carried out in the presence of serum. The growth medium was removed from BASMC that were 40–60% confluent, and 500 μ l of fresh growth medium (including 10% FBS) were added. Triplicate wells were given 1 μ g freshly compacted DNA as a positive control, 1 μ g

of released DNA, or 1 μ g of naked DNA. Negative control wells contained no DNA. After 24 h, the growth medium with the DNA was removed, the cells were lysed, and luciferase activity was quantified.

2.8. Statistical analysis

Results were analyzed using the two-tailed Student *t*-test. A *P*-value <0.05 was employed to indicate statistical significance.

3. Results

3.1. Compaction of plasmid DNA

The luciferase plasmid was successfully compacted using the cationic conjugate, CK₃₀PEG10k/TFA. The plasmid DNA concentration was measured using UV-absorbance before and after compaction. As expected, the concentration was 0.22 mg/ml before compaction and 0.2 mg/ml after compaction. The DNA was then concentrated to over 4 mg/ml. The effects of compaction on DNA morphology were evident on transmission electron micrographs of naked supercoiled (Fig. 1A) and compacted (Fig. 1B) plasmid DNA. Other characterization assays including sedimentation, serum stability, and light scattering (data not shown) demonstrated that the DNA was non-aggregated, stable in serum for 2 h at 37 °C, and compacted into small rods and globules (25 \times 40 nm).

3.2. Gel and MT–Gel delivery system fabrication

The compacted DNA was successfully incorporated into the Gel and MT–Gel systems. The mass of DNA loaded into the microtubules was estimated by calculating the inclusion volume of the microtubules. This has been shown to be an accurate method of quantifying microtubule loading [21]. The average length of the DNA-loaded microtubules was 25.9 \pm 11.6 μ m, and the yield was 3.6 \times 10⁸ tubules per mg lipid, as determined using light microscopy. The expected mass of DNA loaded into 1 mg of lipid microtubules was 5.5 μ g, 4.6% of the DNA initially added (120 μ g). Each sample of the hydrogel and

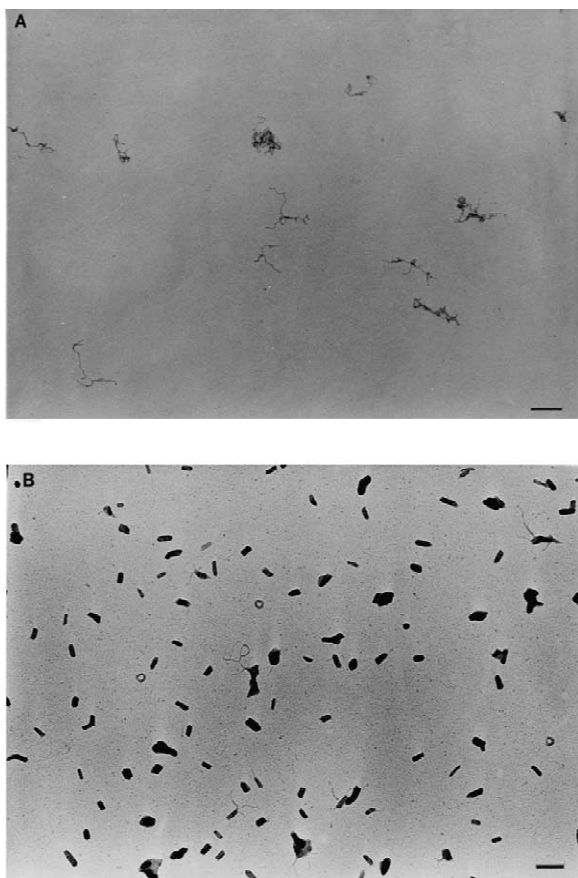


Fig. 1. Transmission electron micrographs of naked supercoiled (A) and compacted (B) DNA. Scale bars=100 nm.

the microtubule–hydrogel delivery system was gelled in a glass vial to form a cylindrical block with a diameter of 11.6 mm and a height of 5.7 mm.

3.3. Characterization of sustained release of compacted DNA

Compacted DNA released from the Gel and MT–Gel systems was quantified via UV-absorbance at 260 nm and plotted as the cumulative percent of DNA released (Fig. 2). The Gel system provided sustained delivery, releasing $64 \pm 5\%$ of the initially loaded DNA after 55 days. The MT–Gel system released at a slower rate, delivering $30 \pm 7\%$ of the DNA loaded into the microtubules after 50 days.

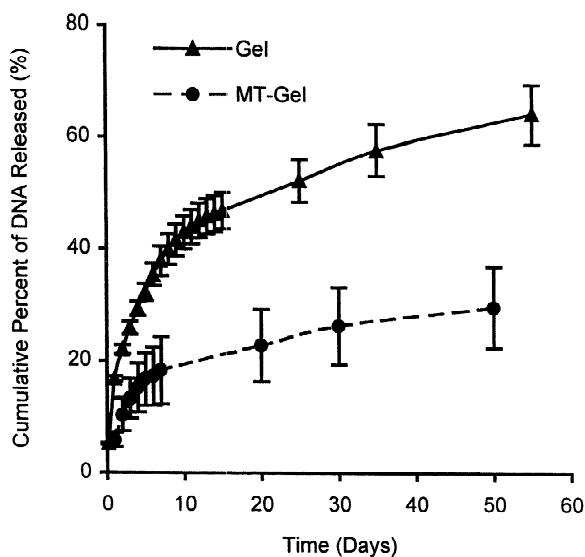


Fig. 2. Release profile of compacted DNA from hydrogel (Gel) and microtubule–hydrogel (MT–Gel) delivery systems. The cumulative release of DNA was plotted as the percent of initially loaded DNA (mean \pm S.E.M. of triplicate samples).

3.4. Structural characterization of released DNA

The sedimentation assay confirmed that the compacted DNA released from the Gel system was stable and non-aggregated in physiological saline solution. After centrifugation, the UV-absorbance of supernatants from the Gel system changed less than 20% with the exception of day 14, which had a change of 23%.

For the MT–Gel system, only half of the supernatants from the DNA-loaded MT–Gel and none of the supernatants from the saline-loaded MT–Gel were within 20% of their original absorbance after centrifugation. While the absorbance values for supernatants from the DNA-loaded MT–Gel were significantly higher than those from the saline-loaded MT–Gel, the absolute changes in UV-absorbance for supernatants from the DNA-loaded and saline-loaded MT–Gel systems were not statistically different (P -value >0.05), except on day 50. The post-spin UV-absorbance was used to determine the concentration of the DNA released from the MT–Gel system in order to remove any microtubule contributions.

Transmission electron microscopy was used to evaluate the DNA released from the Gel system. The

electron micrographs revealed short rods and globules in the supernatants collected on days 1, 25, and 55 (Fig. 3A–C, respectively). These DNA complexes were similar in size and morphology to the freshly compacted DNA (Fig. 1B). DNA released from the MT–Gel system was too dilute to image properly.

3.5. Serum stability assay

Electrophoretic analysis of the serum-incubated DNA samples demonstrated that both the freshly compacted DNA and the DNA released from the Gel and MT–Gel systems were compacted and resistant to degradation by nucleases present in mouse serum (Fig. 4). As expected, untreated freshly compacted DNA (lane 2) did not migrate through the gel, because these neutrally charged particles have an essentially zero zeta potential (data not shown). Likewise, untreated DNA released from the Gel and MT–Gel systems (lanes 5 and 8) did not move into the gel. None of these lanes contained any detectable smearing or degradation.

To analyze the integrity of the plasmid, compacted DNA samples were digested with trypsin to remove the peptide/PEG conjugate. When trypsin-digested, freshly compacted DNA (lane 4) consisted of supercoiled and nicked plasmid forms, whereas released DNA (lanes 7 and 10) was linear and nicked with no detectable supercoiled DNA. No smearing or degradation was detected.

When freshly compacted DNA samples were both serum-incubated and trypsin-digested, a portion of the supercoiled plasmid was converted to either nicked or linear plasmid forms without any DNA degradation (lane 3, compared to lane 4). The released DNA samples that were serum-incubated and trypsin-digested were nicked and linear with no detectable degradation (lanes 6 and 9). Naked DNA, on the other hand, completely degraded to low molecular weight fragments during the serum-incubation (data not shown).

For the released DNA samples, the relative intensities among the lanes cannot be compared, since differing amounts were added to the lanes to maximize the DNA loaded and ensure visualization.

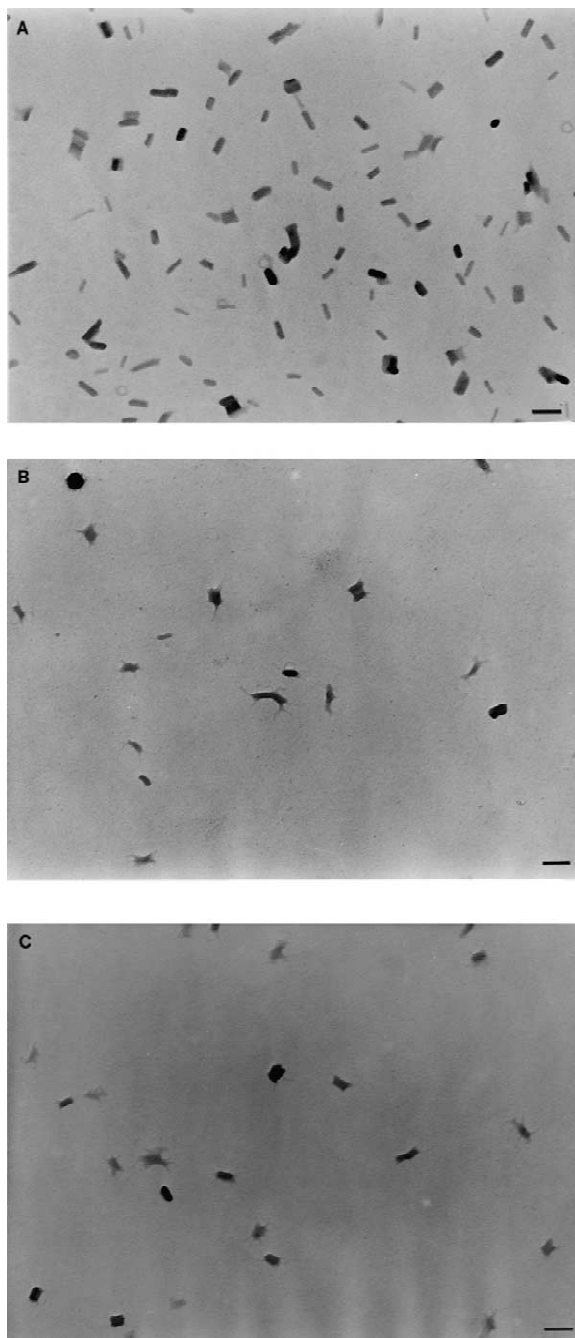


Fig. 3. Transmission electron micrographs of compacted DNA released from the Gel delivery system during day 1 (A), days 15–25 (B), and days 35–55 (C). Scale bars=100 nm.

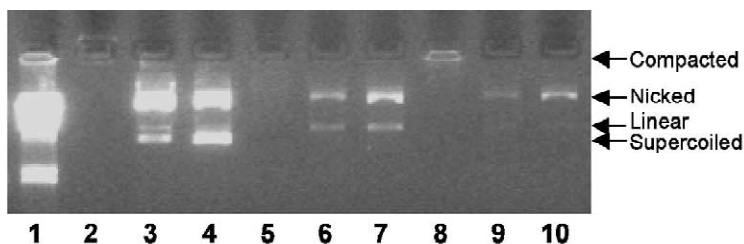


Fig. 4. Analytical gel electrophoresis of freshly compacted and released DNA to visualize serum stability. Lane 1, DNA ladder. Lanes 2–4, freshly compacted DNA that was untreated (lane 2, 1500 ng), serum-incubated and trypsin-digested (lane 3, 250 ng), and trypsin-digested (lane 4, 250 ng). Lanes 5–7, released DNA from the Gel (days 15–25) that was untreated (lane 5, ~480 ng), serum-incubated and trypsin-digested (lane 6, ~48 ng), and trypsin-digested (lane 7, ~250 ng). Lanes 8–10, released DNA from the MT-Gel (days 7–20) that was untreated (lane 8, ~180 ng), serum-incubated and trypsin-digested (lane 9, ~18 ng), and trypsin-digested (lane 10, ~83 ng).

3.6. Functional characterization of released DNA

The DNA released from the delivery systems transfected BASMC in OptiMEM without any additional transfection agents, such as cationic lipids (Fig. 5). Luciferase activity from the freshly compacted DNA and all released DNA samples was significantly above background levels and the activity from naked plasmid DNA. However, activity from the released DNA was lower than that from freshly compacted DNA ($P < 0.05$). Released DNA from other time points resulted in similar gene expression (data not shown).

Transfections were also performed with naked,

freshly compacted, and released DNA in the presence of serum proteins. Both freshly compacted and released DNA (days 1–2) were able to transfect in the presence of 10% serum (Fig. 6), producing significantly higher gene expression than naked DNA ($P < 0.05$). Gene expression from naked plasmid was indistinguishable from background activity. Released DNA resulted in reduced gene expression compared to freshly compacted DNA. DNA released at later times also produced luciferase activity in the presence of serum, with expression levels similar to those of DNA released on days 1–2 (data not shown).

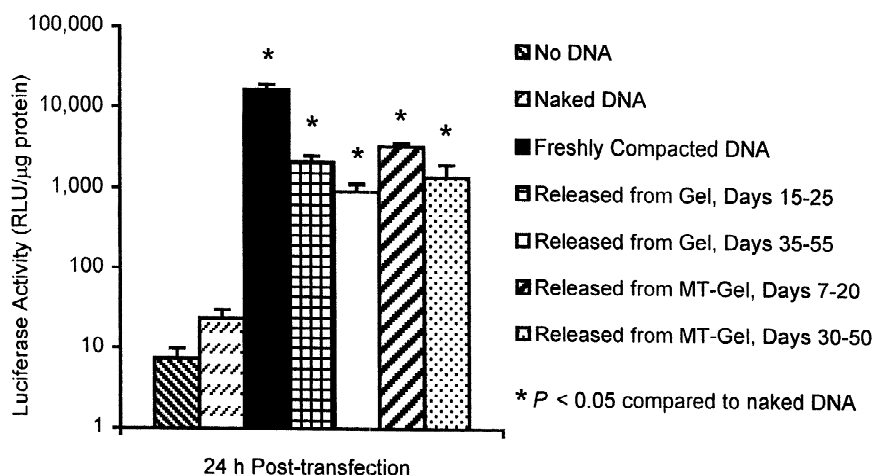


Fig. 5. Effect of sustained release of compacted DNA on its ability to transfect BASMC in reduced-serum medium. Data are expressed as the mean \pm S.E.M. of at least three wells. * P -value < 0.05 compared to naked DNA.

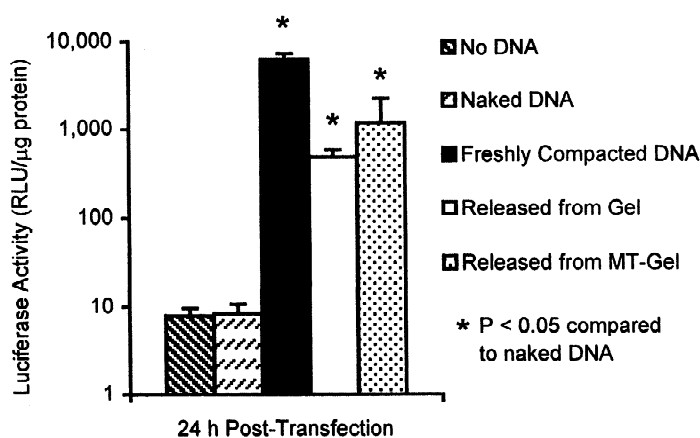


Fig. 6. Effect of serum on the ability of naked and compacted DNA to transfect BASMC. Freshly compacted and released (days 1–2) DNA transfected BASMC in the presence of serum. Data are expressed as the mean \pm S.E.M. of at least three wells. **P*-value < 0.05 compared to naked DNA.

4. Discussion

In this study, the approach to improving non-viral transfections involved: (1) using DNA compacted with a cationic peptide–PEG conjugate to enhance transfection efficiency, and (2) designing a sustained delivery system that retained the structural and functional integrity of released DNA. It was demonstrated that hydrogel and microtubule–hydrogel delivery systems can successfully release compacted DNA that is structurally similar to the unreleased, freshly compacted DNA and functionally capable of transfecting smooth muscle cells *in vitro*. Although the activity of the released DNA was reduced, the transfection efficiency was still significantly greater than naked DNA in both reduced-serum medium (OptiMEM) and regular growth medium with serum.

In designing a delivery system with the potential to prolong non-viral gene expression, agarose hydrogel and lipid microtubules were utilized to release compacted DNA. The diffusive release of DNA from the hydrogel depends upon the DNA concentration gradient, the DNA size and shape, and the agarose geometry. Agarose has an open-cell morphology, and pore size is dependent on the concentration [29]. By changing the agarose concentration, it may be possible to alter the release profile. This gel system is advantageous because it can either be gelled *in situ*

or a pre-gelled solution can be injected into the site of interest (unpublished results).

The MT–Gel system consists of agarose hydrogel and lipid microtubules. Microtubules are hollow, open-ended tubules that self-assemble from the DC_{8,9}PC lipid [30]. Release from the MT–Gel system is a two-step process. The microtubules, confined by the hydrogel, serve as reservoirs for the compacted DNA and slowly release the DNA into the hydrogel. The DNA then diffuses through the pores of the hydrogel into the surroundings.

To augment the transfection efficiency associated with the Gel and MT–Gel systems, a luciferase plasmid was compacted with the CK₃₀PEG10k/TFA conjugate. Structurally, the compacted DNA was concentrated to over 4 mg/ml, non-aggregated in physiological saline, physically smaller than naked DNA (Fig. 1), and protected from nuclease degradation (Fig. 4). The compacted DNA formulations used in this study were essentially non-toxic in formal GLP toxicity studies [31] and were stable for prolonged periods of time at 37 °C. UV-absorbance was an appropriate method to quantify the compacted DNA, since the absorbance of the DNA before and after compaction was as expected.

For *in vitro* transfections, uncompact DNA by itself did not produce luciferase activity above background, whereas freshly compacted DNA trans-

fectected BASMC in reduced-serum medium (Fig. 5) and in the presence of 10% FBS (Fig. 6). Compacted DNA has also been shown to transfect post-mitotic cells in vitro [32] and to produce efficient transfection in vivo [24,25].

Both the Gel and MT–Gel delivery systems released compacted DNA for over 50 days (Fig. 2) with the release profile depending on the system. Although neither system released 100% of the loaded DNA within the 50-day time course of the experiments, it is expected that release will continue until all the DNA is released. Since the gel electrophoresis analysis did not show any plasmid degradation, the unreleased DNA likely remained in the delivery systems. Both the agarose and the microtubules have a slightly negative charge. If the compacted DNA particles, which have a near neutral charge, associate with the delivery system components, the release would be affected. If release from the delivery systems did continue, the mass released at later time points may be insufficient to meet therapeutic needs. Since the optimal amount of DNA may be different in vitro and in vivo, system parameters, such as DNA loading concentration, hydrogel concentration, and microtubule density, can be easily adjusted to customize the delivery systems. In addition, the release profile of the Gel system is more sensitive to the geometry of the formulation than the MT–Gel system, with greater surface area to volume ratios resulting in quicker release (unpublished results). Thus, depending on the geometry and the application, either the Gel or the MT–Gel system can be selected and optimized.

Compacted DNA incorporated into the Gel and MT–Gel systems remained physically compacted and chemically stable upon release, even after prolonged incubation at 37 °C. The sedimentation assay results suggested that DNA released from both the Gel and MT–Gel systems was non-aggregated. Although some supernatants from the MT–Gel system were not within the acceptable range of $\pm 20\%$ for the sedimentation assay, the reduction in UV-absorbance was likely due to microtubule sedimentation and not to DNA aggregation and sedimentation. Microtubules scatter light at 260 nm, and all supernatants from the MT–Gel system contained some microtubules that had loosened from the hydrogel and pelleted during centrifugation. Since there was

no difference in the absolute reduction in UV-absorbance for supernatants from the saline-loaded and DNA-loaded MT–Gel samples, the floating microtubules, and not the DNA, most likely caused the reduction in UV-absorbance for the DNA-loaded MT–Gel.

TEM evaluation confirmed that released DNA from the Gel was structurally intact (Fig. 3). The released DNA from the MT–Gel system was too dilute for TEM visualization. Additional release studies were performed using DNA compacted with CK₃₀PEG10k containing an acetate counterion; these rod-like particles are efficiently released from the MT–Gel system. TEM analysis of this DNA released from the Gel and MT–Gel systems during days 7–20 revealed that the compacted structure was unchanged (data not shown). These data indicate that both the Gel and the MT–Gel systems can incorporate and release structurally intact compacted DNA.

The stability of the released DNA was demonstrated by its resistance to nuclease degradation (Fig. 4). No degradation was detected in any freshly compacted or released DNA samples. Freshly compacted DNA contained a mixture of supercoiled and nicked DNA, but released DNA was primarily nicked with some linear DNA. Other plasmid DNA delivery systems have also converted DNA to a relaxed form after loading and release [5,9,33–35]. While their DNA formulations and delivery systems are different from the Gel and MT–Gel systems, a common observation emerges: conversion of the plasmid DNA from supercoiled to nicked and/or linear forms. EDTA has been shown to reduce the conversion of supercoiled DNA [16] and can potentially be used in conjunction with these delivery systems to increase the supercoiled content of the released DNA.

The released DNA was functional and able to transfect BASMC in reduced-serum medium and in growth medium with serum. However, activity was reduced compared to freshly compacted DNA (Figs. 5 and 6). To determine if the delivery system components were affecting the transfection efficiency, BASMC were transfected with freshly compacted DNA in the presence of whole microtubules, broken microtubules, hydrogel solution, or gelled hydrogel. Neither the hydrogel nor the microtubules had a significant effect on the resulting

luciferase activity (data not shown). The reduced activity may result from the conversion of our released DNA to nicked/linear forms. Other studies on DNA release have also shown that the released DNA had reduced supercoiled content as well as reduced gene expression compared to the original DNA [7,12,33]. This reduced DNA activity could be taken into consideration when designing for long-term release applications.

It is understood that *in vivo*, the duration of expression from non-viral gene therapy can be shortened by a variety of mechanisms, including cell division, cell death, promoter shutdown due to the methylation state of the DNA, promoter inactivation due to cytokine production, and DNA destruction [36]. A sustained release delivery system addresses these issues. Cell division and death, changes in DNA methylation, and promoter inactivation can be overcome by transfecting new cells. If the delivery system protects the DNA from degradation, new DNA can be provided when the available DNA is destroyed. Thus, sustained release systems, such as the Gel and MT–Gel delivery systems, have the potential to overcome many of the potential causes of transient gene expression and improve non-viral gene therapy.

5. Conclusions

Using the Gel and MT–Gel delivery systems, compacted DNA can be released for extended periods of time at 37 °C and can maintain structural and functional integrity. The combination of DNA compaction and slow release provides a promising gene delivery system that may significantly improve the low transfection efficiency and transient gene expression associated with non-viral gene therapy. Due to their versatility, these delivery systems could be used for a number of applications that would benefit from persistent, local gene expression, including cardiovascular disease, skin disease, cancer, and tissue engineering.

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