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Nanospheres Formulated from L-Tyrosine Polyphosphate Exhibiting Sustained Release of Polyplexes and In Vitro Controlled Transfection Properties

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Abstract: Currently, viruses are utilized as vectors for gene therapy, since they transport across cellular membranes, escape endosomes, and effectively deliver genes to the nucleus. The disadvantage of using viruses for gene therapy is their immune response. Therefore, nanospheres have been formulated as a nonviral gene vector by blending L-tyrosine-polyphosphate (LTP) with polyethylene glycol grafted to chitosan (PEG-g-CHN) and linear polyethylenimine (LPEI) conjugated to plasmid DNA (pDNA). PEG-g-CHN stabilizes the emulsion and prevents nanosphere coalescence. LPEI protects pDNA degradation during nanosphere formation, provides endosomal escape, and enhances gene expression. Previous studies show that LTP degrades within seven days and is appropriate for intracellular gene delivery. These nanospheres prepared by water−oil emulsion by sonication and solvent evaporation show diameters between 100 and 600 nm. Also, dynamic laser light scattering shows that nanospheres completely degrade after seven days. The sustained release of pDNA and pDNA−LPEI polyplexes is confirmed through electrophoresis and PicoGreen assay. A LIVE/DEAD cell viability assay shows that nanosphere viability is comparable to that of buffers. X-Gal staining shows a sustained transfection for 11 days using human fibroblasts. This result is sustained longer than pDNA−LPEI and pDNA−FuGENE 6 complexes. Therefore, LTP−pDNA nanospheres exhibit controlled transfection and can be used as a nonviral gene delivery vector.

Keywords: Gene delivery; nonviral; nanospheres; nanoparticles; polyplexes

1. Introduction

In recent years, preclinical studies have highlighted the potential benefits of gene therapy to treat diverse clinical conditions including cancer, cardiovascular disease, numerous monogenic and polygenic diseases, and tissue engineering.[1−3] Gene therapy can provide treatment for both genetically based and acquired diseases by introducing DNA or RNA into cells to restore the cell's normal function.[4] Successful introduction of genetic material can only be achieved by overcoming the body's natural barriers, which include degradation by enzymes, crossing the cellular membrane, and escaping endosome entrapment. Currently, viral vectors are the primary method for delivering genes into host cells and achieving gene expression.[5] Viruses are

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uniquely equipped to encapsulate genes in a capsid to prevent enzyme degradation, bind and enter into the cellular membrane, and escape from endosome entrapment.[6] These unique abilities of the virus led to the first and only successful clinical trial in gene therapy where retroviral vectors were used to introduce the adenosine dearminase gene into the $CD34^+$ cells of patients suffering from severe combined immunodeficiency (SCID).[7] However, viral vectors have disadvantages such as limited DNA loading capacity, high expense, toxicity, immunogenicity, and potential replication of competent viruses.[6] Furthermore, deaths during gene therapy clinical trials have been potentially linked to the viral vectors.[8,9] These unfortunate incidents suggest that a safer vector is needed for gene therapy.

The compelling motivation for the development of nonviral vectors is the safety issues associated with viruses. However, the main drawback of these systems is the poor transfection abilities of DNA.[10,11] In order to increase their effectiveness, DNA has been complexed with either cationic lipids (lipoplexes) or cationic polymers (polyplexes).[10,11] Both lipoplexes and polyplexes can carry large amounts of DNA, but most are toxic at high dosages.[10] In addition, they can only obtain brief transient transfections.[11]

In contrast, synthetic biodegradable polymers can provide a sustained transfection by degrading and releasing pDNA at controlled rates.[12] These vectors can decrease the number of doses and maintain optimum dosage levels for gene expression.[13] Previous studies have used hydrogels, polymer matrices, and microspheres to obtain a controlled release.[14,15] Microspheres formulated from hyaluronan exhibit a sustained release of pDNA over nine weeks, which is ideal for long-term therapies.[15] However, these delivery methods accomplish an extracellular release of genes. DNA delivered to the outside of a cell is unable to achieve high expression due to charge repulsion from cellular membranes, extracellular degradation by enzymes, and intracellular entrapment and degradation by endosomes and lysosomes.[16] Therefore, adequate transfection cannot be accomplished without complexing the genes and/or delivering the genes to the inside of the cell.[17]

Biodegradable nanospheres and nanoparticles hold a unique advantage over other nonviral vectors, since they are comparable in scale to viruses and can be internalized. Particles less than 1 μm can enter most eukaryotic cells by endocytosis.[18] Furthermore, nanospheres are large enough to encapsulate genetic material.[19] In addition, ideal nanospheres for gene delivery vectors should not elicit an immune response, should be biodegradable, should not show cellular toxicity, should protect the genetic material from enzyme degradation, and should be able to escape from endosome encapsulation.[10] Biodegradable nanospheres loaded with DNA have been made from poly[DL-lactide-*co*-glycolide] (PLGA) and hyaluronan, which take several months to degrade.[15,20] Unfortunately, these nanospheres do not degrade fast enough to release all of their genetic material within the cytoplasm of cells that live for only several days.[21] Hence, nanospheres would need to be formulated from a polymer that matches the life cycle of the targeted cells.

Therefore, we have formulated nanospheres using a rapidly degrading polymer. LTP (molecular weight of 8,000 to 11,000 Da) is synthesized from the modification of the natural amino acid L-tyrosine. This polymer degrades by hydrolysis in seven days.[22] Hydrolytic degradation of LTP

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occurs at the phosphoester linkages producing nontoxic L-tyrosine based derivatives and phosphates, along with low amounts of alcohols.[22] Unlike PLGA, the degradation products of LTP have negligible effect on local pH.[23] LTP is soluble in a variety of organic solvents and can be easily processed into nanospheres.[23] The process of formulating LTP into nanospheres has been previously described.[24] In this study, LTP nanospheres have been encapsulated with pDNA complexed with linear polyethylenimine (LPEI) and evaluated for their potential as a gene delivery vehicle.

2. Experimental Section

2.1. Chemicals. All aqueous based reagents used in the following experiments were prepared with distilled and deionized (Barnstead NanoPure II, Dubuque, IA) water (DH_2O) that was autoclaved (American Standard 25X-1) to inactivate DNAase. PEF1/V5-His/LacZ (9.2 kb, Invitrogen, Carlsbad, CA) plasmid DNA was propagated using a QIAGEN plasmid purification kit. LPEI from PolyScience Inc. (Warrington, PA) with a molecular weight of 25,000 Da was dissolved in DH_2O at 70 °C at a concentration of 3 mg/mL. Polyethylene glycol grafted to chitosan (PEG-g-CHN, CarboMer Inc., San Diego, CA, 80% acetylation) was dissolved at a concentration of 3.33 mg/mL in 0.1 N acetic acid for 48 h at 37 °C under rotation. Polyvinylpyrrolidone (PVP, Sigma-Aldrich, St. Louis, MO) was dissolved in DH_2O at a concentration of 5%. PLGA (inherent viscosity of 0.59 dL/g in HFIP at 30 °C, Absorbable Polymers International, Pelham, AL) was dissolved in dichloromethane (DCM, Emanuel Merck, Darmstadt, Germany) at a concentration of 100 mg/mL. Heparin (Sigma-Aldrich, St. Louis, MO) was dissolved in DH_2O at a concentration of 30 mg/mL.

2.2. LPEI and pDNA Complex. Before encapsulation, pDNA was complexed to LPEI. The polyplex was formed by incubation of 3 mg of pDNA with 3 mg of LPEI at 37 °C in 10 mL of DH_2O for 45 min. The polyplex was used immediately for the pDNA nanosphere formulation.

2.3. Nanosphere Synthesis. LTP−pDNA nanospheres were prepared using an emulsion of water and oil by sonication and solvent evaporation technique. Nanosphere formulations shown in Table 1 were emulsified using a sonicator (Branson 102C CE, Danbury, CT) for 1 min. Nanosphere synthesis was performed in 6 replicates. Blank nanospheres, noncomplexed pDNA nanospheres, and blank PLGA nanospheres were formulated as negative controls (Table 1). Next, the chloroform was allowed to evaporate for 5 h while the emulsion was gently stirred. The nanospheres were then collected and washed three times with DH_2O by centrifugation at 15000*g* for 15 min. Afterward, the nanospheres were shell frozen in 10 mL of DH_2O and lyophilized (Labconco Freezone 4.5, Kansas City, MO) for 72 h. Finally, the lyophilized nanospheres were stored in a desiccator.

Table 1. Nanosphere Formulations

polymer	concn (mg/mL)	vol (mL)	mass (mg)	mass % (w/w)	vol % (v/v)
LTP−pDNA Nanospheres					
LTP	100.0	2.91	291.0	97.0	2.8
pDNA−LPEI	0.6	10.00	6.0	2.0	9.6
PEG-g-CHN	3.3	0.90	3.0	1.0	0.9
5% PVP		90.00			86.7
total		103.81	300.0		
Blank Nanospheres					
LTP	100.0	2.94	294.0	97.0	2.8
LPEI	3.0	1.00	3.0	2.0	0.9
PEG-g-CHN	3.3	0.90	3.0	1.0	0.9
5% PVP		100.00			95.4
total		104.84	300.0		

Table 2. pDNA Uncomplexing from LPEI with Heparin

	initial concn (μg/μL)	vol (μL)	mass (μg)	final concn (μg/μL)	heparin:pDNA mass ratio
pDNA−LPEI (pDNA)	0.05	16.0	0.8	0.008	2500:1
heparin	30.00	68.0	2004.0	20.400	2550:1
DH_2O		16.0			
total		100.0	2004.8		2550:1

2.4. Scanning Electron Microscopy of LTP−pDNA Nanospheres. Scanning electron microscopy (SEM, Hitachi S2150, Japan) was used in order to qualitatively compare the size, shape, and morphology of LTP−pDNA nanospheres to the blank LTP nanospheres. Nanospheres (1 mg) were suspended in 1 mL of DH_2O. Then, 200 μL of the suspended nanospheres was pipetted onto a stub, dehydrated, sputter coated with silver/palladium, and examined.

2.5. Characterization of Nanospheres' Size, and Degradation. Dynamic laser light scattering (DLS, Brookhaven Instruments BI-200SM, Holtsville, NY) was used to quantify the sizes of the LTP−pDNA nanospheres. The nanosphere samples were prepared by suspending 1 mg of nanospheres in 10 mL of phosphate buffered saline (PBS, pH 7.4) that had been passed through a 0.2 μm filter. The suspended nanospheres were centrifuged for ten seconds at 1000*g* to remove any large aggregates. Then, the sample was decanted into a glass scintillation vial. The DLS system calculated the nanosphere diameter by the regularized non-negatively constrained least squares (CONTIN) method. The range of nanosphere size was reported as differential distribution values. The differential distribution value varied from 0 to 100. The highest peak or modal value was assigned to the number 100 and reported as relative amounts.

In order to determine the degradation of the nanospheres in vitro, the nanospheres were incubated at 37 °C under constant rotation for seven days after the initial light scattering measurement. On days 1, 2, 3, 4, and 7, DLS was performed. The mean diameter of nanospheres was calculated for each day as previously reported.

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2.6. Loading Efficiency of LTP−pDNA Nanospheres. The loading efficiency of the nanospheres was determined with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). The LTP−pDNA nanospheres (2 mg) were dissolved in 0.2 mL of chloroform for one hour at 37 °C. Then, an equal volume of TE buffer was added and lightly shaken for two minutes. The phase separation between the chloroform and the TE was allowed to form for 30 min. This mixture was then centrifuged for 5 s at 10000*g*. Next, 200 μL of TE supernatant was sampled and incubated with Quant-iT PicoGreen fluorescence dye. The amount of pDNA was determined according to manufacturer's instructions[25] and referenced to a standard curve composed of dilutions of pDNA−LPEI polyplexes in TE buffer.

2.7. Agarose Gel Electrophoresis of pDNA−LPEI Released from Nanospheres. The release of pDNA and/or polyplexes from nanospheres was characterized using gel electrophoresis. LTP−pDNA nanospheres (2 mg) were suspended in 500 μL of TE buffer and incubated at 37 °C under constant rotation. At time points of 0.5, 1.5, 3.0, 6.0, and 12.0 h and 1, 2, 3, 4, 5, 6, and 7 days, the nanosphere suspensions were centrifuged at 10000*g*, and 450 μL of the supernatant was collected and replaced with an equal volume of fresh TE buffer. Next, the release samples were lyophilized and resuspended in 100 μL of TE buffer. Finally, the structural integrity of the pDNA and/or polyplexes released from the nanospheres was analyzed by agarose gel electrophoresis. Supernatant from each of the release samples (30 μL) was mixed with 6 μL of 6× loading dye and loaded into a 0.8% agarose gel containing ethidium bromide.

2.8. Release Profile of pDNA from Nanospheres. In order to characterize the release profile of pDNA from the nanospheres, the pDNA was decomplexed from the LPEI with heparin and quantified using the Quant-iT PicoGreen dsDNA assay kit.[25] Since LPEI complexing to pDNA decreases the fluorescence of the PicoGreen assay, decomplexing from the LPEI is accomplished by incubation of the release samples with heparin. The decomplexing of the pDNA from the LPEI allows the fluorescence dye to interact with the pDNA. The release samples (16 μL) were incubated with heparin for 15 min at room temperatures with a LPEI: heparin mass ratio of 1:2550 in amounts specified in Table 2. Next, the decomplexed pDNA was quantified according to the manufacturer's procedure. Known standards of pDNA−LPEI complexes were decomplexed with heparin and used to calculate amounts of pDNA released from the LTP−pDNA nanospheres.

2.9. Transfection Efficiency of Nanospheres. To qualitatively examine the transfection efficiency of LTP−pDNA nanospheres, an X-Gal (Mediatech, Inc., Manassas, VA) assay was performed on primary human dermal fibroblasts (a gift from Judy Fulton at the Kenneth Calhoun Research Center, Akron General Medical Center). The X-Gal staining was used to determine the percentage of cells transfected with pDNA expressing β-galactosidase (β-gal). In this assay, the product of the lacZ gene, β-gal, catalyzed the hydrolysis of X-gal, producing a blue color within the cell.[27] The transfection percentage was calculated by dividing the number of blue cells by the total number of cells, which determined the transfection efficiency of the nanospheres. Fibroblasts were seeded onto 24 well tissue culture plates at a density of 25,000 cells/well and maintained overnight at 37 °C with fibroblast feeding media that consisted of 90% Dulbecco's modified Eagle medium (DMEM, Mediatech, Inc., Manassas, VA), 10% fetal calf serum (Mediatech, Inc., Manassas, VA), and 1% antibiotic−antimycotic solution (Mediatech, Inc., Manassas, VA). The next day, the fibroblast feeding medium was replaced. Next, LTP−pDNA and blank nanospheres were suspended in feeding medium and added to the wells seeded with fibroblasts to obtain a final concentration of 0.67 μg/μL (400 μg) of nanospheres in the well. As a control, fibroblasts were transfected with a mixture of 200 ng of stock pDNA (100 ng/μL), 1.9 μL of FuGENE 6 (Roche, Germany), and 96.1 μL of DMEM. After 3, 5, 7, 9, and 11 days of incubation, the fibroblasts were fixed with 1% formaldehyde in PBS for ten minutes. The X-Gal assay was performed according to the manufacturer's instructions. For each transfection ($n = 3$ for each time point), three random fields were selected using a microscope (Axiovert 200, Carl Zeiss, Peabody, MA) with a 20× magnification lens, and bright field images were captured using a Cannon Power Shot G5 camera. Blank nanospheres, 4 μg of complexed pDNA with LPEI, 4 μg of complexed pDNA with FuGENE 6, blank TE buffer, and 4 μg of pDNA were used controls.

2.10. Cell Viability after Exposure to Nanospheres. The cell viability of primary human dermal fibroblasts after exposure to LTP−pDNA nanospheres was determined using a LIVE/DEAD cell viability assay kit (Invitrogen, Carlsbad, CA). Cell viability was recorded as the percentage of live cells per total cells. Nanospheres, which fluoresced green with the same filter as dead cell nuclei, were distinguished by their smaller diameters and more spherical shape as opposed to the typical morphology of normal nuclei. Primary human dermal fibroblasts were seeded onto 24 well tissue culture plates at a density of 25,000 cells/well and maintained overnight at 37 °C with fibroblast feeding media. The next day, the fibroblast feeding medium was replaced. Each well of fibroblasts with 500 μL of feeding medium was exposed to 400 μg of nanospheres suspended in 200 μL of feeding medium. After 1, 3, 7, and 11 days a LIVE/DEAD cell viability assay was performed according to the manufacturer's instructions. For each treatment ($n = 3$ for each time point), three random fields were visualized using fluorescence microscopy (Axiovert 200, Carl Zeiss, Peabody, MA) at 200× magnification and images were captured using a CCD camera (AxioCam HRm, Carl Zeiss, Peabody, MA). Blank

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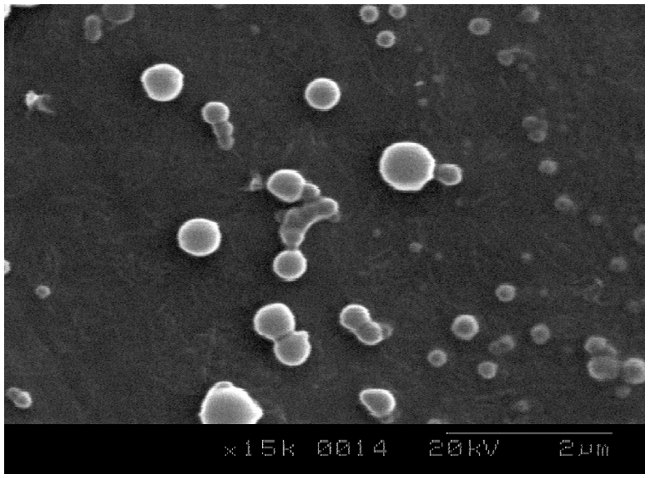

Figure 1. SEM of LTP−pDNA nanospheres (15000× magnification).

nanospheres, pDNA−LPEI, pDNA−FuGENE 6 complex, blank PLGA nanospheres, pDNA, and TE buffer were used as controls.

2.11. Statistics. All quantitative studies were performed in 6 replicates determined by power analysis with $\alpha = 0.05$. The Shapiro−Wilk test for normality was performed for each sample group.[26] Samples were considered normally distributed when $p \leq 0.05$. Sample data were considered continuous since they were calculated mean values.[27] Tukey's analysis of variance was then performed among the normally distributed sample groups. All results were considered significant if $p \leq 0.05$.

3. Results

3.1. SEM of LTP−pDNA Nanospheres. SEM is utilized to examine the nanospheres' morphology, size, and shape. These images reveal a smooth surface for the LTP–pDNA nanospheres. The shapes of LTP−pDNA nanospheres are generally spherical (Figure 1).

3.2. Characterization of Nanospheres' Size, and Degradation. DLS shows that the diameter range of LTP−pDNA nanospheres is between 128 and 716 nm (Figure 2). This result is comparable to the diameter range of blank LTP nanospheres in previous studies.[24] The encapsulation of pDNA−LPEI complexes in the nanospheres appears to have little effect on the diameter of the nanospheres.

DLS has been further utilized to characterize the degradation of the nanospheres. After seven days of incubation in PBS at 37 °C, the mean diameter has been reduced down to 40 nm, which indicates progressive mass loss from the degradation of the nanospheres upon exposure to an aqueous solution (Figure 3). The mean diameter of 40 nm is consistent with the size of the

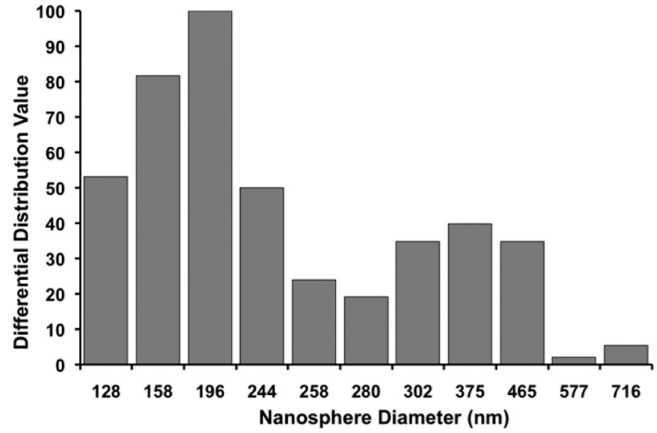

Figure 2. Representative size distribution of LTP−pDNA nanospheres determined using regularized non-negatively constrained least squares (CONTIN).

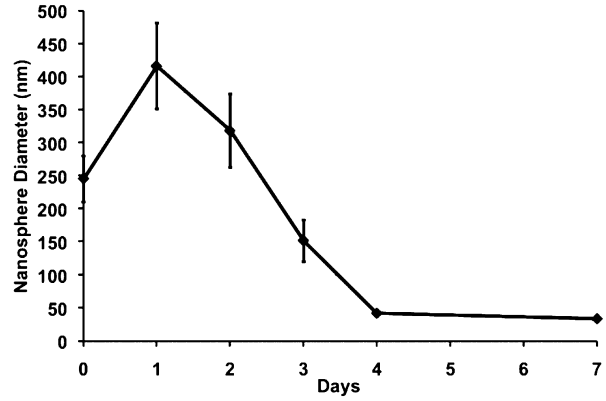

Figure 3. Degradation of LTP−pDNA nanosphere mean diameter determined using regularized non-negatively constrained least squares (CONTIN).

pDNA−LPEI complex.[28] This degradation profile is comparable to previous studies with blank LTP nanospheres and LTP films, which are completely degraded in PBS at 37 °C after 7 days.[23,24] The mean diameter of the blank nanospheres decreased by 75% after 4 days of incubation in PBS at 37 °C (Figure 3). However, the degradation profile of the LTP– pDNA nanospheres has an unexpected increase in the mean diameter on day 1 (Figure 3), which may be due to nanosphere aggregation.

3.3. Loading of pDNA−LPEI into Nanospheres. The encapsulation efficiency of pDNA into the nanospheres is determined using the Quant-iT PicoGreen dsDNA assay kit. Since a decrease in fluorescence is observed when pDNA is complexed with LPEI, a standard curve for emission fluorescence and concentration is made using titrations of the pDNA−LPEI complexes.[29] The loading

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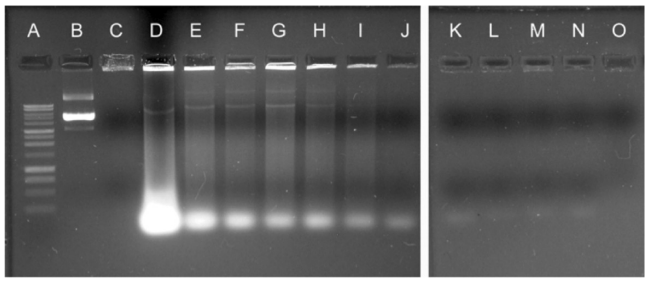

Figure 4. Gel electrophoresis of (A) 1 kb DNA ladder, (B) stock pDNA, (C) stock pDNA−LPEI, LTP−pDNA nanosphere release after (D) 0.5 h, (E) 1.5 h, (F) 3.0 h, (G) 6.0 h, (H) 12.0 h, (I) 24 h, (J) 2 days, (K) 3 days, (L) 4 days, (M) 5 days, (N) 6 days, (O) 7 days.

of pDNA is then determined by dividing the mass of the pDNA by the total nanosphere mass. The encapsulation efficiency of LTP−pDNA nanospheres is 40 ± 3%, which corresponds to a 0.4% (w/w) loading of pDNA into the nanospheres.

3.4. Agarose Gel Electrophoresis of pDNA−LPEI Released from Nanospheres. Agarose gel electrophoresis is performed in order to qualitatively characterize the release of the LTP−pDNA nanospheres. When pDNA is complexed with cationic polymer LPEI at a 1:1 mass ratio (corresponds to 7.7:1 nitrogen:phosphate ratio), the charge is negated and the complex does not migrate through the gel, which is consistent with previous data.[6] Gel electrophoresis reveals that a release of pDNA−LPEI is sustained over seven days (Figure 4). The greatest release of pDNA−LPEI appears between 0.5 h to the first day (Figure 4). Bands of pDNA−LPEI are still visible between days 2 and 7, but the UV fluorescence grows fainter with each passing time point (Figure 4). Noncomplexed supercoiled pDNA and relaxed pDNA are seen in the released samples for 0.5 h to day 2 (Figure 4). High to low molecular weight streaks in the gel are determined to be pDNA sheared from sonication and are observed in the released samples for 0.5 h to day 2 (Figure 4).

3.5. Release Profile of pDNA Nanospheres. The release profile of the LTP−pDNA nanospheres has been generated from a Quant-iT PicoGreen dsDNA assay kit after decomplexing the pDNA from LPEI with heparin. The cumulative release profile shows a total of 3 μg of pDNA released from 1 mg of nanospheres after 7 days. This cumulative release corresponds to the pDNA loading study (Figure 5), since no significant difference exists between the amount of pDNA found in the loading study and the cumulative release of pDNA nanospheres ($p = 0.073$). Approximately 70% of the pDNA is released in the first 4 days, which mimics the LTP degradation rates.[23,24]

3.6. Transfection Efficiency of Nanospheres. The controllable and sustained transfection from nanospheres has been demonstrated by X-Gal staining of human dermal fibroblasts exposed to 400 μg of LTP−pDNA nanospheres. The cumulative transfection profile using LTP−pDNA nanospheres demonstrates an initial delay until day 5, and sustained transfection

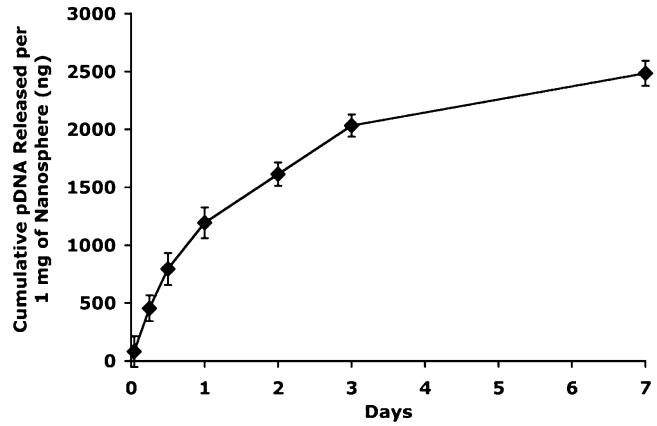

Figure 5. Cumulative release of pDNA from LTP−pDNA nanospheres obtained with PicoGreen quantification of pDNA after heparin decomplexation.

is observed after the fifth day (Figures 6−8). No significant differences are observed among transfection percentages of the LTP−pDNA nanospheres on days 7, 9, or 11 ($p = 0.995$, $p = 0.829$, $p = 1.00$ respectively). The transfection percentage of LTP−pDNA nanospheres on days 3, 5, 7, 9, and 11 are respectively 1 ± 1%, 4 ± 1%, 10 ± 1%, 8 ± 1%, and 6 ± 1%. No significant differences in transfection percentage are found between LTP−pDNA nanospheres and pDNA−LPEI polyplexes for days 5, 9, and 11 ($p = 0.9989$, $p = 0.177$, and $p = 0.286$) and pDNA−FuGENE 6 lipoplexes for days 5, 9, and 11 ($p = 1.00$, $p = 0.317$, $p = 0.899$). However, the transfection percentage for LTP−pDNA nanospheres on day 3 is found to be significantly different ($p < 0.001$) than pDNA−LPEI and pDNA−FuGENE 6. In addition, LTP−pDNA nanospheres on day 7 have a significant increase in transfection compared to pDNA−LPEI ($p = 0.015$) and pDNA−FuGENE 6 ($p = 0.012$). Fibroblasts exposed to 4 μg of pDNA after 3, 5, 7, 9, and 11 days demonstrate no detectable transfection, which is comparable to buffers alone (Figure 8).

Unlike LTP−pDNA nanospheres that demonstrate a controlled and sustained transfection, pDNA complexes with LPEI and FuGENE 6 have diminishing transient transfection profiles (Figure 8). Exposing fibroblasts to 4 μg of pDNA complexed at a 1:1 mass ratio with LPEI achieves a high transfection percentage of 25 ± 5% after 3 days (Figure 8). However, the transfection percentage diminishes after the fifth day to 5 ± 1%, 2 ± 1%, 2 ± 1%, and 2 ± 1% on days 5, 7, 9, and 11 respectively (Figure 8). A diminishing transfection percentage is also observed for pDNA complexed with FuGENE 6. The highest transfection efficiency using FuGENE 6 occurs at day 3 with a 15 ± 2% transfection. Similar to LPEI, the transfection efficiency of FuGENE 6 decreases after day 5, 7, 9, and 11 to 5 ± 2%, 2 ± 1%, 3 ± 1%, 3 ± 1% (Figure 8).

3.7. Cell Viability after Exposure to Nanospheres. The viability of human dermal fibroblasts after an 11 day exposure to LTP−pDNA nanospheres is determined using a LIVE/DEAD cell viability assay. In Figure 10 (in the

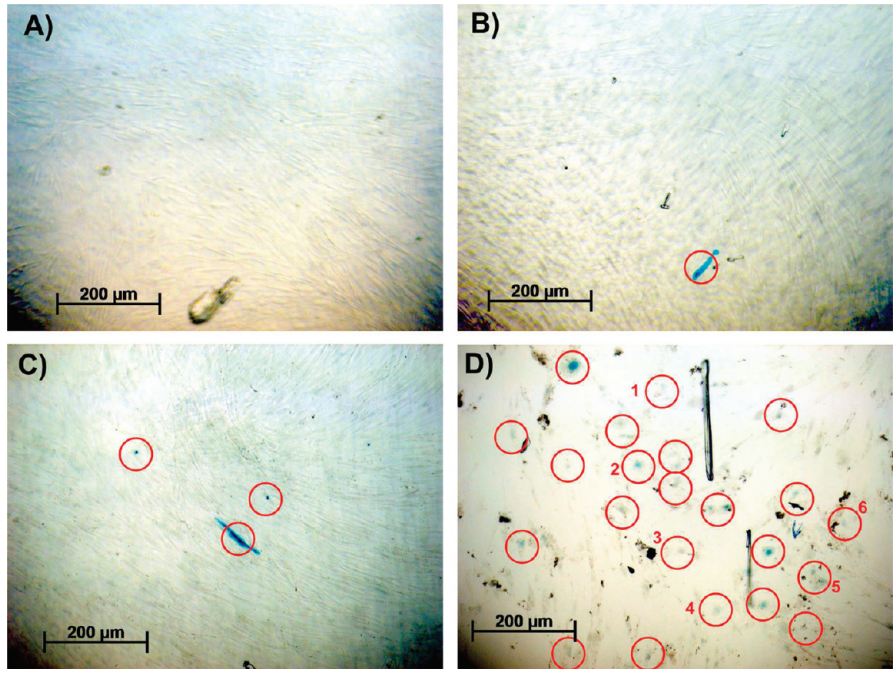

Figure 6. Bright field images (100× magnification) of day 7 of human dermal fibroblasts transfected with (A) 4 μg of pDNA, (B) 4 μg of pDNA with 12 μL of FuGENE 6 transfection reagent, (C) 4 μg of pDNA complexed with 4 μg of LPEI, (D) 400 μg of LTP−pDNA nanospheres.

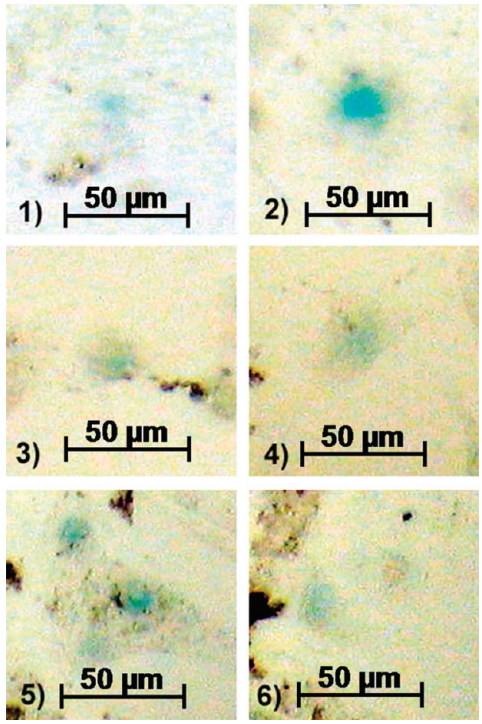

Figure 7. Close-up view of bright field images of selected human dermal fibroblasts transfected after 7 day exposure to LTP−pDNA nanospheres. (Numbers correspond to Figure 6.)

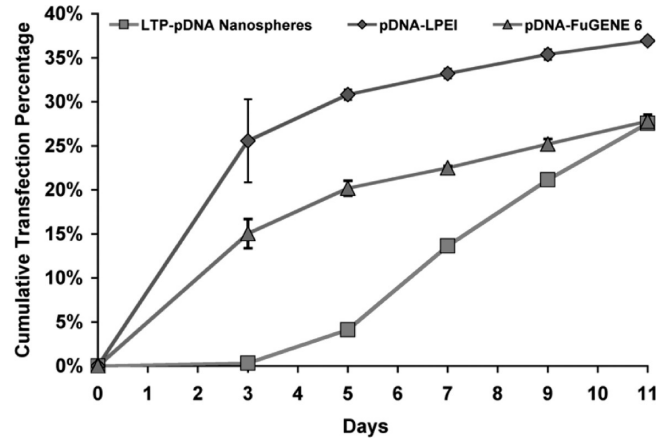

Figure 8. Cumulative transfection efficiency profile of LTP−pDNA nanospheres versus pDNA−LPEI and pDNA−FuGENE 6 over 11 days.

Supporting Information), the dead cells are circled to improve their visualization. Fibroblasts exposed to 400 μg of LTP−pDNA nanospheres after 11 days have a viability of 91 ± 1% (Figures 9 and 10 in the Supporting Information). Similar results are seen for TE buffer, 4 μg of pDNA, and PLGA nanospheres that respectively result in fibroblast viabilities of 91 ± 1%, 91 ± 1%, and 88 ± 7% (Figure 10 in the Supporting Information), which are not significantly different from LTP−pDNA nanospheres ($p = 1.00$). However, lower viabilities are obtained with exposure to 4 μg of pDNA complexed to LPEI, 400 μg of blank nanospheres, and 4 μg of pDNA complexed to FuGENE 6 that yield viabilities of 78 ± 6%, 84 ± 4%, and 63 ± 4%, respectively (Figure 10 in the Supporting Information). Fibroblast viabilities after 11 days of exposure to LTP−pDNA nanospheres were significantly different than blank nanospheres ($p < 0.001$), pDNA−FuGENE 6 ($p < 0.001$), and pDNA−LPEI ($p = 0.001$).

4. Discussion

The development of nonviral vectors has become necessary due to the potential risks for immunological complications with viruses.[30] Current alternatives to viruses include cationic lipoplexes and polymeric micro/nanoparticles.[31] However, cationic lipoplexes are highly toxic and quickly cleared from the body.[28,29] For most depot delivery systems such as biodegradable micro- and nanoparticles, the degradation rates are too slow for efficient intracellular release of the DNA.[15,31] In addition, these polymer micro/nanoparticles release naked pDNA, which increases its persistence but does not improve DNA's poor transfection ability. Furthermore, the released DNA after endocytosis does not possess the means to escape from endosomal entrapment. To address this weakness, we have formulated nanospheres as a nonviral gene vector that incorporate the endosomal escape technology of LPEI[11,32] with the rapid degradation of LTP.[23,24]

We have formulated our nanospheres with the necessary size for endocytosis, which results in an intracellular delivery of pDNA that increases transfection efficiency by avoiding enzyme degradation in the circulation.[12] In order to be endocytosed, the nanospheres must mimic the scale of viruses by being less than 1 μm in diameter. Typically, eukaryotic cells can internalize nanoparticles with diameters ranging from 50 nm to 1 μm.[33] Results from SEM and DLS demonstrate that our LTP−pDNA nanospheres possess the appropriate size, 100 to 700 nm, for cellular uptake (Figures 2 and 3). Furthermore, previous studies show that LTP nanospheres of similar size can be internalized by human fibroblasts.[24]

Endocytosed nanospheres (or nanoparticles) must degrade within the life span of cells, which can range from 1 day to years, in order to deliver their entire pDNA load within the cell.[21] For nanospheres formulated from biodegradable polymers such as PLGA and LTP, the targeted cells must be able to divide. Since the degradation rate has been linked to the efficacy of nonviral gene delivery systems,[34] PLGA nanoparticles, which have a degradation half-life in the order of months, are too slow for an intracellular delivery of DNA.[35] For LTP−pDNA nanospheres, DLS data shows complete degradation after 7 days (Figure 4). Furthermore, the release profile of LTP nanospheres shows a pDNA release over a period of 7 days (Figure 5). This observed sustained release profile is a result of a rapid hydrolytic degradation of LTP[23] and is ideal for intracellular delivery.

While several viral vectors such as retrovirus can incorporate their DNA into the host's genome,[36] nonviral vectors can only achieve a transient expression of their genes before they are cleared.[37,38] Studies have shown that nonviral vectors such as cationic polyplexes and lipoplexes can maintain gene expression for only 24 to 72 h.[38] We have extended the gene expression of nonviral vectors by utilizing the sustained release technology of our degradable nanospheres. The X-gal staining reveals that pDNA nanospheres achieve a sustained transfection for at least 11 days in human fibroblasts, while gene expression with LPEI and FuGENE 6 diminishes after 72 h (Figures 6−8). This sustained transfection is likely a result of a controlled release of pDNA−LPEI complexes from LTP nanospheres. We hypothesize that the cumulative transfection percentage could be increased by combining the LTP−pDNA nanospheres with free LPEI-pDNA complexes, which could be tested in future studies.

By complexing pDNA with LPEI before encapsulation, LPEI neutralizes the negative charge of the pDNA, protects their integrity during nanosphere formation, and provides our gene delivery vector an escape from endosomal entrapment. The release of noncomplexed pDNA in extracellular fluid or cytoplasm has poor transfection efficiency due to its inability to pass through cellular membranes, escape entrapment in endosomes, and lysosomal degradation.[28,39,40] Since the cellular membrane has a negative charge, pDNA is repelled and consequently cannot pass through. Complexing with LPEI neutralizes the overall charge of pDNA and condenses pDNA into complexes of approximately 20 to 50 nm diameters.[11]

The complexing of pDNA with LPEI also protects pDNA from shearing during the formation of emulsion. Sheared

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pDNA is no longer bioactive due to the loss of its structural integrity.[40] The gel electrophoresis data demonstrates that a population of pDNA released from the nanospheres is still complexed to LPEI (Figure 4). The integrity of these pDNA−LPEI polyplexes is verified, since the release products are found to be bioactive in the X-gal staining transfection studies (Figures 6−8).

Furthermore, LPEI has been shown to provide a route of escape from endosomes and prevent lysosome degradation by the "proton sponge theory".[41] The amino groups in the backbone of LPEI possess a low pK_a, buffer the lysosome,[6,11] induce an increased osmotic pressure within endosomes, and cause endosomal swelling and bursting.[6,11] Once released into the cytoplasm, the pDNA−LPEI complex is available to enter the nucleus during mitosis.[41] However, the exact mechanism by which polyplexes enter the nucleus is unknown at this time. LPEI and pDNA are believed to dissociate and interact with RNA polymerase in order to achieve gene expression.[42] Other studies have shown diffusion of polyplexes through nuclear pores, but their diameters must be less 25 nm.[43]

In addition, LPEI and PEG-g-CHN have been shown to stabilize emulsions, which help the formation of nanospheres by preventing aggregation and increasing yield.[44] The amphiphilic properties of PEG-g-CHN and LPEI are comparable to other copolymers such as poly(ethylene glycol) ethyl ether methacrylate,[45] which are used to stabilize oil and water emulsions. In our nanosphere fabrication, amphiphilic PEG-g-CHN collects at the water and oil interface,[46] which prevents coalescence of nanoparticles due to PEG's steric stabilization properties.[47] Furthermore, PEG and chitosan are ideal for incorporation into a nonviral vector since they have been shown to be nontoxic as well.[48]

In order to have an advantage over viral vectors, nonviral gene delivery systems and their degradation products must be nontoxic. Nonviral vectors such as cationic lipoplexes and cationic polymers such as linear and branched polyethylenimine (BPEI) have been shown to be toxic.[49,50] However, a LIVE/DEAD cell viability assay has demonstrated that our pDNA nanospheres possess toxicity comparable to buffers and pDNA during the sustained transfection time period (Figures 9 and 10 in the Supporting Information). These results correspond to toxicity studies performed with empty LTP nanospheres.[24] The lack of toxicity of our LTP−pDNA nanospheres is due to titration of DNA and LPEI complex based upon the N/P ratios.[50] The only potentially nonbiocompatible degradation products from the nanospheres are LPEI and hexanol. LTP's covalent ester bond of the hexanol hydrolyzes, but does not break as readily as the phosphoester bond in the backbone, which leads to the primary degradation of LTP.[23] In addition, LPEI is released at a controlled rate and is complexed with pDNA, which titrates its toxic effects to safer levels.[50] Hence, the local concentration of hexanol and LPEI should not reach toxic levels.[13,25]

The development of safe vectors has become a necessity for the advancement of gene therapy as a reliable means to treat cancer, cardiovascular disease, numerous monogenic and polygenic diseases, and to achieve tissue regeneration. LTP nanospheres loaded with pDNA−LPEI polyplexes have been formulated as nonviral gene vectors. These nanospheres demonstrate a sustained gene expression. A sonication created the oil−water emulsion, and solvent evaporation proves to be an effective method to create LTP−pDNA nanospheres without damaging the pDNA's bioactivity. Complexing pDNA with LPEI protects the pDNA from shearing during nanosphere fabrication. Furthermore, LPEI provides escape from endosomal entrapment and lysosomal degradation,[39] which enhances our vector's transfection. The addition of PEG-g-CHN into the nanospheres stabilizes the emulsion. The LTP−pDNA nanospheres are spherical, smooth, and appropriate size for endocytosis. In addition, these nanospheres degrade in an ideal time frame for an intracellular release of pDNA and/or polyplexes due to LTP's rapid degradation rate. This prolonged release accomplishes a sustained transfection for at least 11 days, which is unlike the brief

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transfection rates for other nonviral vectors. Furthermore, our LTP−pDNA nanospheres are nontoxic to human fibroblasts in vitro, which should lead to favorable biocompatibility in vivo. Therefore, our nanospheres could be used as a nonviral vector for gene therapy that requires controlled and prolonged transfections.

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Supporting Information Available: Figure 9 depicting LIVE/DEAD cell viability assay and Figure 10 consisting of a plot of cell viability of various gene vectors as a function of number of days. This material is available free of charge via the Internet at http://pubs.acs.org.

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