Published in final edited form as:

Mol Pharm. 2013 May 6; 10(5): 1836–1844. doi:10.1021/mp300623a.

***In Vivo* Gene Delivery with L-Tyrosine Polyphosphate Nanoparticles**

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Abstract

The concept of gene therapy is promising; however, the perceived risks and side effects associated with this technology have severely dampened the researchers' enthusiasm.^{1–3} Thus, the development of a non-viral gene vector without immunological effects and with high transfection efficiency is necessary. Currently, most non-viral vectors have failed to achieve the *in vivo* transfection efficiencies of viral vectors due their toxicity,⁴ rapid clearance,^{5,6} and/or inappropriate release rates.⁷ Although our previous studies have successfully demonstrated the controlled-release of plasmid DNA (pDNA) polyplexes encapsulated into nanoparticles formulated with L-tyrosine polyphosphate (LTP-pDNA nanoparticles),^{8,9} the *in vivo* transfection capabilities and immunogenicity of this delivery system has yet to be examined. Thus, we evaluate LTP-pDNA nanoparticles in an *in vivo* setting via injection into rodent uterine tissue. Our results demonstrate through X-gal staining and immunohistochemistry of uterine tissue that transfection has successfully occurred after a nine-day incubation. In contrast, the results for the control nanoparticles show similar results to shams. Furthermore, reverse transcriptase polymerase chain reaction (RT-PCR) from the injected tissues confirms the transfection *in vivo*. To examine the immunogenicity, the LTP nanoparticles have been evaluated in a mouse model. No significant differences in the activation of the innate immune system are observed. These data provide the first report for the potential use of controlled-release nanoparticles formulated from an amino acid based polymer as an *in vivo* non-viral vector for gene therapy.

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Supporting Information

Result includes a representative RT-PCR gel for the rat uterus injected with LTP nanoparticles. (1) 100bp ladder, (2) LTP nanoparticle (no pDNA-LPEI), (3) LTP-pDNA nanoparticle, and (5) No template control. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

Keywords

Nanoparticles; L-tyrosine; Gene therapy; Non-viral; Immune response

INTRODUCTION

Advances in the field of non-viral gene therapy have been the subject of intensive research because of the inherent safety issues associated with most viral vectors.^{10, 11} Numerous strategies for increasing transfection efficiencies have been developed. These strategies include the complexing of DNA with lipids (lipoplex) or cationic polymers (polyplex) or the encapsulation of DNA and/or polyplexes into nanoparticles composed from biodegradable polymers, liposomes, or micelles. Despite these and other advances of non-viral delivery vehicles, *in vivo* transfection efficiency that rivals most viral vectors has remained elusive.^{12, 13}

Key characteristics of viruses that are attributed to their efficiency in gene transduction include their ability to protect genetic material within capsids, to recognize cellular receptors, to transport genetic material across cellular membranes, to escape from endosomes, and to enter into the nucleus using an active transport mechanism.^{13, 14} Many of these viral features can be synthetically replicated into various polymeric platforms, such as nanoparticles. Our nanoparticles (Figure 1) are formulated from LTP, a non-toxic biodegradable polymer.¹⁵ This polymer degrades into desaminotyrosine, L-tyrosine, phosphates, and alcohols and has a negligible effect on pH.^{8, 15, 16} The degradation rate of LTP polymer is 7 to 10 days *in vitro*, which is an appropriate time frame for intracellular delivery of genetic materials.^{8, 15}

The viral features incorporated into LTP-pDNA nanoparticles include the encapsulation of pDNA complexed with linear polyethyleneimine (LPEI) to simulate the endosomal escape through the proton sponge theory. The size LTP-pDNA nanoparticles ranges from 100 to 700 nm, which is suitable for transport across the cellular membrane by endocytosis; and the surface has been decorated with polyethylene glycol (PEG) to aid in the evasion of the immune system, which is a key feature of retroviral vectors.^{8,9} Furthermore, the PEG on the surface of the LTP-pDNA nanoparticles can be easily conjugated with targeting moieties for cellular specificity.¹⁷

In an *in vitro* setting, LTP-pDNA nanoparticles have shown promise as a gene delivery device. Our lab previously has demonstrated that the size of LTP-pDNA nanoparticles prepared using an oil-water emulsion technique range from 100 to 700 nm in diameter.⁸ The nanoparticles have smooth and spherical morphology.^{8, 15} The loading efficiency for LTP-pDNA nanoparticles is 0.4% (w/w) of which a significant population of pDNA remains structurally intact during the nanoparticle preparation process.⁸ Additionally, the degradation time period for the LTP polymer is approximately seven days and the entire content of the pDNA-LPEI complex is released from the nanoparticles during this period.^{8, 15} Confocal microscopy demonstrates that primary human dermal fibroblasts in culture are able to uptake blank nanoparticles made with LTP (LTP nanoparticles).⁸ The overall transfection of these cells is equal to primary human dermal fibroblasts exposed to pDNA complexed with Fugene 6.⁸ Unlike LPEI polyplexes that show peak transfection after three days, LTP-pDNA nanoparticles exhibits controlled transfection between 5 to 11 days of incubation.^{8, 15}

To develop and translate LTP-pDNA nanoparticles as an alternative to viral vectors for therapeutic applications, an essential step is the *in vivo* evaluations of the transfection capabilities as well as immunogenic responses of the host. In addition, our laboratory has

been interested in the potential use of these nanoparticles as a treatment for a variety of diseases, such as preeclampsia, and the uterus provides a unique and highly metabolic tissue to study the potential of LTP nanoparticles as a gene delivery device. For this study, LTP-pDNA nanoparticles have been investigated for their *in vivo* transfection abilities using a rat uterus model as well as their potential to induce inflammatory and innate immune system responses.

EXPERIMENTAL SECTION

Animals for LTP-pDNA transfection

Wistar-Kyoto female virgin rats were obtained from a colony housed at the University of Akron. At the start of the experiments, the ages of these animals were 10–14 weeks. They were paired house in standard polyethylene cages (45 × 25 × 20 cm) with stainless steel tops. All cages consisted of heat-treated wood chip bedding (PJ Murphy, Montville, NJ) with standard chow (Teklad Rodent, Madison, WI) and water *ad libitum*. Room temperature was held constant about ~25°C with ~40% humidity and a 12 hour light (07:00–19:00)/dark (19:00–07:00) cycle. All protocols were reviewed and approved by the Institutional Animal Use and Care Committee (IACUC) at the University of Akron.

Synthesis of LTP-pDNA Nanoparticles

LTP-pDNA nanoparticles were prepared using an emulsion of water and oil by sonication and solvent evaporation technique as previously described by Ditto et al.⁸ LTP was synthesized according to the protocol developed by Sen Gupta.¹⁸ PEF1/V5-His/LacZ (9.2 kb, Invitrogen, Carlsbad, CA) plasmid DNA was propagated using a QIAGEN plasmid purification kit. LPEI (PolyScience Inc., Warrington, PA) with a molecular weight of 25,000 Daltons was dissolved in autoclaved and deionized water (DH₂O) at 70°C at a concentration of 1 mg/ml. Immediately before nanoparticle encapsulation, pDNA was complexed to LPEI at a 1:1 mass ratio by incubating at 37°C in 10 ml of DH₂O for 45 minutes.

A typical batch of LTP-pDNA nanoparticles consisted of 3 mL of 100 mg/mL of LTP in chloroform, 0.9 mL of 3.33 mg/mL polyethylene glycol grafted to chitosan (PEG-g-CHN) in 0.1M acetic acid, 10 mL of 0.6 mg/mL pDNA-LPEI (complexed prior to emulsification), and 100 mL of 10% (w/v) polyvinylpyrrolidone, which was emulsified with a sonicator (Branson 102C CE, Danbury, CT) for 1 minute. Next, chloroform was allowed to evaporate for 5 hours while the emulsion was gently stirred. The nanoparticles were then collected and washed three times with DH₂O by centrifugation at 15,000xg for 20 minutes. Afterwards, the nanoparticles were shell frozen in DH₂O and lyophilized (Labconco Freezone 4.5, Kansas City, MO) for 72 hours. Finally, the lyophilized nanoparticles were stored in a desiccator until use.

Control nanoparticles were produced using identical formulations with the exception of appropriate variations. For LTP nanoparticles, pDNA-LPEI was substituted with Tris-EDTA buffer. The poly(lactic-co-glycolic acid) (PLGA, 50/50, 0.95 – 1.20 dL/g, LACTEL Absorbable Polymers, Birmingham, AL) nanoparticles encapsulated with pDNA-LPEI were produced by substituting LTP with PLGA.

Injection Protocol for Transfection Studies

Animals (n = 4 per group) were anesthetized with 2% Isoflurane (Viking Medical) in 100% oxygen at a flow rate of 1L/min. A midline incision was made and the uterus was exposed. Nanoparticles were delivered as an injection of 100 μ L of 25 mg/mL solution (the total amount of pDNA injected was 10 μ g per animal) in sterile saline with a 0.5cc insulin syringe (28 gauge) into the myometrium of the left horn of the uterus. Equivalent amounts

of LTP-pDNA, PLGA-pDNA, and LTP nanoparticles were used for the injection protocols. Sham animals were operated upon but not injected and used as non-injection controls.

Termination and Uterus Isolation

Nine days post injection, animals were terminated with an overdose of 2.5% Sodium Pentothal (Eli Lilly & Co., Indianapolis, IN). The uterus was excised and the left horn was cut in half with one uterine piece flash frozen in liquid nitrogen within 3 minutes of termination and stored at -80°C for RT-PCR analysis. The other half of the uterus was placed in 1% buffered formalin for transfection and H & E analysis.

Cryosectioning

Uterine horn samples fixed in 1% buffered formalin were washed overnight (4°C) with sterile phosphate buffered saline (PBS, pH 7.4) and then cryoprotected overnight (4°C) with 30% sucrose. Tissues were embedded in Optimal Cutting Temperature (OCT; EMS, Hatfield, PA) compound and sectioned with a Bright Model OTF cryosection (Bright Instrument, Huntingdon. Cambs., England) at 50 micron thickness. Slices were stored at -80°C until tissue analysis.

β -gal Staining

Tissue sections were permeabilized with 1mM MgCl_2 , washed with sterile PBS and incubated with X-gal solution for a period of three days. Tissue sections were covered from light and placed in a CO_2 incubator at 37°C for the duration of the incubation. β -gal staining of the tissue was imaged with microscopy (Axiovert 200, Carl Zeiss, Peabody, MA) at 100X, 200X, and 630X magnification, and images were captured using a CCD camera (AxioCam ICc3, Carl Zeiss, Peabody, MA). The tissues were counterstained with hematoxylin for contrast.

Immunofluorescence Assay

Tissue sections were subjected to immunofluorescence analysis. Mouse monoclonal antibody against the *E. coli* expressed β -gal (Promega, Madison, WI) was directly placed (1:50 dilution) on tissue sections overnight (4°C). Tissues were washed with sterile PBS and then incubated with polyclonal anti-mouse IgG-TRITC secondary antibody (Sigma Aldrich, St. Louis, MO, 1:50 dilution) overnight at 4°C .

The tissue samples that were stained for β -gal with the TRITC secondary antibody were imaged with a Zeiss 510 META laser-scanning module on an Axiovert microscope (Carl Zeiss Inc., Peabody, MA) with a 543nm laser excitation wavelength and a 40x oil-immersion objective. In order to resolve the TRITC probe fluorescence from the tissue auto-fluorescence, emission reference spectra were taken from both a non-stained tissue section and from the TRITC conjugated secondary antibody, spanning an emission wavelength from 548.6nm to 643.1nm with 10.7nm steps. Using the LSM 510 software, these reference spectra were saved to a database file. For the stained tissue sections, lambda stacks were again taken from 548.6nm to 643.1nm at 10.7nm steps. The fluorescence from the TRITC probe was unmixed from the autofluorescence of the tissue using the reference spectra, and the signals assigned separate colors (green for tissue auto-fluorescence and red for TRITC). Control images were also taken for tissue sections from the LTP nanoparticle injected rats stained with TRITC secondary antibody and for sham tissue sections.

Hematoxylin and Eosin Staining

Formalin fixed tissue was sectioned using a cryosection at 50 μm sections and subjected to Hematoxylin and Eosin (H & E) staining. Tissue sections were then dehydrated and

mounted for Bright field imaging. Histological analysis of tissue morphology, inflammatory and leukocyte markers were assessed with microscopy (Axiovert 200, Carl Zeiss, Peabody, MA) with 10X, 20X, and 63X objectives, and images were captured using a CCD camera (AxioCam ICc3, Carl Zeiss, Peabody, MA).

Reverse Transcriptase Polymerase Chain Reaction

Frozen tissues were homogenized under liquid nitrogen using mortar and pestal. Tissue powder was then further homogenized using a tissue homogenizer with Trizol reagent (Invitrogen, Carlsbad, CA). RNA isolation was performed according to manufacturer's instructions. RNA was removed of any plasmid DNA or DNA contaminates by treatment with DNase I (1U/ μ L; Roche) and an RNA cleanup kit (Qiagen, Valencia, CA). Purified RNA was stored at -80°C until analysis.

RT-PCR was performed with the following primers: Forward: 5'-ACCCGCATTGACCCTAAC-3', Reverse: 5'-TGTATCGCTCGCCACTTC-3'. A one-step RT-PCR kit (Qiagen) was used according to manufacturer's instructions. The protocol was modified to 30 cycles with a denaturing cycle of 94°C (30 sec), an annealing cycle of 53°C (1 min), and an extension cycle of 68°C (1 min). Products from the RT-PCR reaction were analyzed on a 1% agarose gel with an amplicon of 234 base pairs indicating successful transfection. The amplicons have been previously sequenced to ensure that it was from β -gal origin.¹⁹

Animals for Evaluating the Innate Immune Response

Female C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Mice were 8 to 12 weeks of age when used in all experiments. As in the rat studies, mice were housed and cared as previously described and treated in accordance with the University of Akron's IACUC.

Injection Protocol for the Mice Studies

Complete Freund's adjuvant was purchased from Difco Laboratories (Detroit, MI). It was prepared for injection by adding equal volumes of adjuvant and pyrogen-free PBS then mixed with an 18g needle and glass syringe until homogenous slurry was present. Groups of mice ($n = 3$ per group with 3 replicates) were injected in the hind footpads. Animals received either 400 μg of LTP nanoparticles in 30 μl of pyrogen free sterile PBS (pH 7.2), 30 μl of complete Freund's adjuvant (positive control), or PBS (negative control). Two days after injection, the bone marrow (BM) cells were harvested and cultured *in vitro* with the indicated reagents.²⁰ After another 2 days, the culture media was assayed for nitric oxide (NO) production.

Biological Reagents used to Evaluate Immune Response

The tissue culture media for BM cells consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 10 mM HEPES, 5% fetal calf serum (GIBCO, Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Recombinant murine interferon gamma (IFN- γ) was purchased from ProSpec (East Brunswick, NJ). Lipopolysaccharide (LPS) from *Salmonella typhosa* (Sigma, St. Louis, MO) was dissolved in RPMI pH 7.2 at 1mg/ml and frozen at -80°C .

Preparation of Bone Marrow Cells

Bone marrow (BM) cells were prepared as previously described.²⁰ Briefly, mice were sacrificed, and the femurs and tibias removed. BM cells were flushed with cold Hanks Balanced Salt Solution (HBSS) using a 22g needle and syringe. Red blood cells were lysed

using tris-buffered ammonium chloride and washed 3 times in HBSS. Before culturing, adherent cells were removed by suspending them in tissue culture medium at approximately 5×10^6 cells/mL, placing into a plastic tissue culture flask, and incubating for 1 hour at 37° C and 6% CO₂. After incubation, the non-adherent cells were removed, centrifuged, placed into tissue culture medium and counted. Afterwards, LPS, IFN- γ and LTP nanoparticles were added alone or in combination at the concentrations indicated in Tables 1 and 2. The final volume was adjusted to 1ml with tissue culture medium. Plates were incubated for 48 hours at 37° C, 6% CO₂. After incubation, the plates were spun in the centrifuge (200 x g) for several seconds to ensure that the cells were at the bottom of the plate. Tissue culture supernatants were immediately tested for NO production.

NO Assay

The Griess assay was used to detect the presence of NO.²¹ This assay reduces NO to nitrite, which is detected with a spectrophotometer. Standard curves were generated with each experimental assay. Supernatant were added at 50 μ l to a 96-well plate (Costar, Lowell, MA), with an equal volume of fresh Griess reagent. The reaction was incubated for 5 minutes at room temperature, and analysis was performed using a Dynatech MR 5000 plate reader. All studies were run in triplicate, and statistical analysis was performed using a two-tailed T-test assuming equal variances.

RESULTS

β -galactosidase Staining of *IN VIVO* Rat Uterus Transfection

β -galactosidase (β -gal) staining of the cryosectioned rat uterus injected with nanoparticles has been utilized to demonstrate successful transfection. After 9 days of incubation, LTP-pDNA nanoparticles exhibit high number of transfected cells in the rat uterine horn as indicated by the positive cell staining (shown in blue when printed in color) with X-gal reagent (Figures 2A –2C). These tissues have been counterstained with hematoxylin to highlight the transfected cells (blue cells). Figure 2 also illustrates uterine horn samples injected with LTP (Figures 2D – 2F) and PLGA-pDNA (Figures 2G – 2I) nanoparticles subjected to β -gal staining. These controls show the lack of positive β -gal expression in the tissue. No transfected cells are detectable in the tissue injected with LTP nanoparticles. Only a single transfected cell is observed for PLGA-pDNA nanoparticles.

Confocal Fluorescent Microscopy of *In Vivo* Rat Uterus Transfection

Confocal fluorescent microscopy of cryosectioned rat uterus is used to further demonstrate successful transfection. Immunofluorescence assays demonstrate that LTP-pDNA nanoparticles accomplished *in vivo* transfection 9 days after injection in the rat uterine horn as indicated by the red fluorescence (shown in color print) with the primary antibody against β -gal and secondary TRITC labeled antibody (Figures 3A – 3C). The signals of TRITC and the autofluorescence (shown in green) have been analyzed and separated using the spectral detector of the confocal microscope. Thus, the fluorescence images of the TRITC probe only shows the expression of β -gal. The transfection is most prominent within 15 μ m of the injection site and attenuates as the distance increases from the injection site. In contrast, control uterine horn samples injected with LTP nanoparticles (Figures 3D – 3F) and shams (Figures 3G – 3I) only show autofluorescence (green) of the tissue.

RT-PCR Analysis of *In Vivo* Rat Uterus Transfection

Nine days post-injection, uterine horn tissues have been harvested for RT-PCR analysis. Positive signal of β -gal origin are seen in the rat uterine horns injected with LTP-pDNA nanoparticles (Figure 4, Lane 4). In contrast, no template RNA (Figure 4, lane 2), thoracic

aorta (internal animal control, Figure 4, lane 3), and LTP nanoparticles (see Supporting Information) are lacking transcripts. A 234 base pair amplicon, as expected, is present for stock β -gal pDNA (Lane 5), which serves as a positive control.

Hematoxylin & Eosin Staining of *In Vivo* Rat Uterus Tissue

Hematoxylin and eosin (H & E) staining of cryosectioned rat uterus has been performed to examine the tissue for signs of necrosis, inflammation, and leukocyte activity from the LTP-pDNA nanoparticles. H & E staining has revealed that immune responses to LTP-pDNA nanoparticles (Figures 5A – 5C) are comparable to the LTP nanoparticles (Figures 5D – 5F) and shams (Figures 5G – 5I). The tissue morphology shows no signs of inflammation, and minimal leukocyte activity are observed 9 days after injection. In addition, H & E results for the stained uterine sections show normal nuclei and cellular structures for all samples.

In Vivo Evaluation of the Innate Immune Response

Our investigation has quantified the production of NO using BM cells as a means of examining the influence of nanoparticles on activation of the innate immune system *in vivo*. After the injection of LTP nanoparticles into the hind footpads of mice as this tissue is where the lymphatic system connects to the bone marrow,²² BM cells have been isolated and assayed for NO production. The levels of NO between BM cells harvested from mice injected with LTP nanoparticles and mice injected with PBS show no significant differences ($p = 0.73$). In contrast, mice injected with complete Freund's adjuvant (positive control) exhibit significantly elevated levels of NO production ($67.7 \pm 1.3 \mu\text{M}$, $p < 0.001$) as compared to the LTP nanoparticle injected group ($25.3 \pm 1.6 \mu\text{M}$). Visual inspection of the draining popliteal and inguinal lymph nodes shows no difference in size between the LTP nanoparticle and control groups.

Since the BM cells of animals injected with LTP nanoparticles and PBS show minimal NO production, these cells have been subjected to an additional challenge to further investigate the properties of LTP nanoparticles and the innate immune system. The harvested BM cells have been exposed to additional LTP nanoparticles or in combination with LPS and/or IFN- γ . Table 2 shows that the combinations of LTP nanoparticles with LPS or LTP nanoparticles with IFN- γ show minimal NO stimulation ($2.5 \pm 1.3 \mu\text{M}$ and $1.7 \pm 1.3 \mu\text{M}$, respectively). These results are similar to BM cells harvested from mice injected with PBS ($p = 0.15$ and 0.64 , respectively). However, once BM cells were exposed to the cocktail of LTP nanoparticles, LPS, and IFN- γ , the NO production increases over 12 fold as compared to the LTP nanoparticles alone. This increase in NO production is also apparent for the PBS group. Thus, a detectable inflammation response from the BM cells exposed to LTP nanoparticles require the stimulation of both LPS and IFN- γ in combination.

DISCUSSION

The therapeutic benefits of gene therapy cannot be realized unless the delivery vehicles prevent the immunological complications associated with most viral vectors while maintaining high levels of gene expression.²³ Although many alternatives, such as cationic lipoplexes, polyplexes, liposomes, micelles, polymeric micro/nanoparticles, and others, have been explored because they are relatively safe, none have shown *in vivo* transfection efficiencies that are comparable to viral vectors.^{7, 19, 24, 25} These results could also be attributed to limited strategies employed for improving gene expression. For example, most non-viral delivery systems have focused upon replicating a limited number of viral functions responsible for their success in gene expression, such as protecting DNA through complexation or encapsulation and enhancing the transport of the DNA across the cellular membrane.

In contrast, our strategy is to replicate many of the key viral functions responsible for their enhancing the transfection abilities of nanoparticles. In addition to protection of the encapsulated DNA, the size distribution,^{15, 19} surface pegylation,^{5, 6} incorporation of LPEI,^{26, 27} the choice of the polymer,⁴ and its degradation rates⁷ have been carefully chosen to enhance the gene transfection abilities of LTP-pDNA nanoparticles and to give this vehicle more of the viral-like functions that previously published nanoparticles have possessed.^{19, 28–33} While not all of the components have been added so far, the transfection efficacy of the rat uterus is notably effective in contrast to PLGA-pDNA nanoparticles (Figure 2).

The uterus rat model is an intriguing candidate for the LTP nanoparticle transfection studies due to the tissue's rapidly renewing nature³⁴ and future therapeutic applications.³⁵ In rodents, the uterus is a duplex organ consisting of two separate uterine horns connected at the cervix with collateral blood vessels throughout its length. On the cellular level, each uterine horn consists of an endometrial inner layer of columnar epithelia cells and a myometrial layer of smooth muscle. The rat uterus is a unique physiological organ in that it cycles through a highly metabolic cellular state every 4–5 days.³⁶ Even with the high cellular turnover rate, research with non-viral gene delivery vectors, such as cationic liposome-DNA complexes have shown some success in transfecting the rodent uterus^{34, 37} and oviduct,³⁸ although the transfection is nominal in all of these studies. In contrast, the LTP-pDNA nanoparticles injected into the myometrium of the rat uterus demonstrate that most of the cells in the myometrial smooth muscle layer and in glandular epithelium uptake the nanoparticles with ease, translate the genetic sequence LacZ, and produce a non-native enzyme. The transfection results are observed nine days post injection and suggest that the transfected cells expressed the genetic message over the course of rat's estrus cycle and allow for sustained gene transfection during a highly metabolic and high cell turnover physiological state. All non-viral gene delivery vectors, to date, are most effective for cells that have high rate of mitosis although other vectors lack the effectiveness of the LTP-pDNA nanoparticles *in vivo* for the uterus.^{34, 37} Near the injection site, the majority of tissue is consistently transfected, and positively stained tissue can be detected as far away as 27.5 μm (Figures 2 and 3). However, the attenuation for transfection away from the site of inject is consistent with other studies,^{39–41} as nanoparticles of greater than 100 nm in diameters cannot diffuse freely within the interstitial space.^{42, 43} This observation may also suggest a limited cellular transport mechanism, and the transfection of the tissue remains localized within the uterus, as the LTP-pDNA nanoparticles or the released pDNA did not migrate into the systemic circulation as evidenced by the lack of mRNA expression of β -gal in the thoracic aorta (Figure 4).

As an advantage over viral vectors, LTP-pDNA nanoparticles do not seem to elicit an immune response *in vivo*, which is a major concern with viruses. Previous clinical trials with viral vectors have failed due to complications with the body's immune response to the vectors.^{44–46} However, several non-viral delivery systems, such as polylysine polyplexes and cationic lipoplexes, have been shown to activate the immune responses at comparable levels.^{47, 48} In contrast, our studies show that LTP nanoparticles (loaded with pDNA or blank) lack immunogenicity (Figure 5, Tables 1, and Table 2). H & E staining of LTP-pDNA nanoparticles reveals tissue morphology that is similar to LTP nanoparticles and shams. Histological analysis of the tissue sections exhibits no apoptotic products or inflammatory markers with only minimal leukocyte visualization in all tissue groups.

Further evidence for the lack of immunogenicity from LTP nanoparticles is supported by mouse studies investigating the activation of the innate immune system. The injection of an inflammatory agent, such as complete Freund's adjuvant with LPS or in combination with LPS and IFN- γ , into the footpads of mice results in a measurable activation of the innate

immune system that is apparent 48 hours post injection (Table 1).⁴⁹ This response is manifested by a swelling of the draining lymph node, a release of cytokines, and an increase in NO production by the BM cells. Cytokines, such as IL-6 and IL-17, elicit a rapid immune response and increase the NO production by granulocyte differentiation antigen 1 positive (GR1+) BM cells.^{50–52} In our study, LTP nanoparticles did not appreciably increase the NO production from BM cells either after their injection into mice or by adding them directly to the cultures of BM cells. The LTP nanoparticles, by themselves, resulted in $< 1 \mu\text{M}$ production of NO, which is in stark contrast to the group that contained both LPS and IFN- γ . These results, although only one measure of the activation of innate immunity, suggest that the LTP nanoparticles lack sufficient immunogenicity *in vivo*. However, comparisons with other non-viral as well as viral vectors must be examined in future studies to determine the extent of these findings.

CONCLUSIONS

In conclusion, LTP-pDNA nanoparticles may provide a non-viral alternative vector for the delivery of therapeutic genes *in vivo* to treat diseases, such as preeclampsia and other types of hypertension. Previous data from our laboratory have shown that LTP-pDNA nanoparticles are successful in transfecting human dermal fibroblasts over a sustained period *in vitro*.⁸ However, the successful transfection of cells in an *in vivo* setting and the lack of immunogenicity exhibited by these nanoparticles remain elusive until now. These studies demonstrate that LTP-pDNA nanoparticles encoding for the *E. coli* expressed β -gal gene have successfully transfected the uterus in an *in vivo* rat model. Furthermore, a mouse model provides an initial examination of the favorably low immunogenicity of the LTP nanoparticles. Our investigation provides important and meaningful evidence for the modification of amino acids for preparing controlled-release nanoparticles (as opposed to polyplexes) as a successful gene delivery device *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful for the funding of this research, which was made possible in part through the National Institute of General Medical Sciences (1R01 GM086895), the National Science Foundation (CAREER Award to Yang H. Yun, CBET-0954360), and the University of Akron (Firestone Fellowship to Yang H. Yun and Integrated Biosciences Research Grant to John J. Reho). We also thank Dr. Ronald Salisbury, Associate Professor in the Department of Biology at the University of Akron, for his histological expertise.

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Figure 1. Schematic of the LTP-pDNA and PLGA-pDNA nanoparticles. The image shows a nanoparticle encapsulated pDNA-LPEI complexes and the surface decorated with PEG as the result of the preparation technique.

Figure 2.

Representative brightfield images of β -gal expression in rat uterus sections. The scale bars represent 50 μ m for all images. Uterus injected with (A–C) LTP-pDNA nanoparticles with 10X, 20X, and 63X objectives shows the presence of cells stained with X-gal, which is cleaved by the enzyme β -gal and is converted into a blue-colored product. Thus, the tissue injected with LTP-pDNA nanoparticles shows positive expression of β -gal. Rat uterus injected with (D–F) blank LTP and (G–I) PLGA-pDNA nanoparticles lack the expression of β -gal.

Figure 3.

Representative confocal fluorescent images of *in vivo* rat uterus sections. The scale bars represent 50 μ m for all images. The sectioned uterus was stained with anti- β -gal mouse primary Ab followed by anti-mouse IgG-TRITC secondary Ab. Uterus injected with LTP-pDNA nanoparticles at tissue section depths of (A) 2.5 Pm, (B) 15 Pm, and (C) 27.5 Pm shows gene expression of β -gal. Rat uterus injected with blank LTP nanoparticles or sham controls at tissue section depths of 2.5Pm (D and G), 15 μ m (E and H), and 27.5 Pm (F and I) lack β -gal expression.

Figure 4.

Representative RT-PCR from the rat uterus injected with LTP-pDNA nanoparticles. Tissues were harvested 9 days post-injection. (1) 100 base pair ladder, (2) No template RNA control, (3) Thoracic aorta control (internal animal control), (4) LTP-pDNA nanoparticles for β -gal, and (5) β -gal pDNA positive control.

Figure 5. Representative brightfield images of H & E stained rat uterus sections. The scale bars represent 50 μm for all images. Uterus injected (A–C) with LTP-pDNA nanoparticles at various magnifications shows no detectable damage to tissue and comparable inflammation and leukocyte activity to rat uterus injected with (D–F) blank LTP nanoparticles and (G–I) PLGA-pDNA nanoparticles at various magnifications.

Table 1Quantification of NO [μ M] production from BM cells that have been exposed to LTP nanoparticles *in vivo*

	LTP nanoparticles	PBS	Complete Freund's adjuvant	P values
Tissue culture medium	< 1	< 1	< 1	
LPS, 2.5 μ g	< 1	< 1	23.0 $\pm$ 1.6	
IFN- γ , 5 units	< 1	< 1	< 1	
LPS, 2.5mg	25.3 $\pm$ 1.6	22.4 $\pm$ 1.4*	67.6 $\pm$ 1.3	0.73*
+ IFN- γ , 5 units				< 0.001

Mice have been injected in the footpad with 400 μ g of nanoparticles + PBS, PBS (negative control), or complete Freund's adjuvant (positive control). After 48 hours the BM cells are removed and cultured with the indicated reagents. After culture, the media has been assayed for NO via the Greiss assay. The control groups have been compared to mice injected with LTP nanoparticles.

Table 2

Quantification of NO [μ M] production from harvested BM cells, which have been primed and exposed to additional LTP nanoparticles.

	LTP nanoparticles	PBS	P values
Nanoparticles (NP), 5 μ g	< 1	< 1	
NP + LPS, 2.5 μ g	2.5 $\pm$ 1.3	< 1	0.150
NP + IFN- γ , 5 units	1.7 $\pm$ 1.3	2.0 $\pm$ 1.6	0.640
NP + LPS, 2.5 μ g, + IFN- γ , 5 units	12.7 $\pm$ 1.6	14.1 $\pm$ 1.3	0.280

Mice have been primed with the injection of 400 μ g of LTP nanoparticles + PBS or PBS (negative control) through the footpad. After 48 hours the BM cells are removed and cultured with the indicated reagents. After culture, the media has been assayed for NO via the Greiss assay. The control group is compared to the results of the mice injected with LTP nanoparticles.