Published in final edited form as:

J Control Release. 2008 July 2; 129(1): 66–72.

High Loading Efficiency and Tunable Release of Plasmid DNA Encapsulated in Submicron Particles Fabricated from PLGA Conjugated with Poly-L-lysine

Jeremy S. Blum¹ and W. Mark Saltzman^{1,*}

¹ Department of Biomedical Engineering, Yale University, New Haven, CT

Abstract

Poly(lactic-co-glycolic acid) (PLGA) particles have been widely explored as vehicles for delivery of plasmid DNA to mammalian cells both *in vitro* and *in vivo*. Achieving high incorporation efficiencies and control over release kinetics are significant challenges in encapsulating hydrophilic molecules such as DNA within submicron particles fabricated from PLGA. This study explored two modifications in the preparation of submicron particles to specifically address these challenges. Firstly, we compared homogenization and sonication as energy sources for emulsification. It was demonstrated that particles prepared with homogenization resulted in higher encapsulation efficiency and a linear release profile of DNA as compared to particles prepared with sonication, which exhibited lower encapsulation efficiency and a burst release. Also investigated was conjugation of poly-L-lysine to PLGA (PLGA-PLL) to create an electrostatically favorable interaction between the carrier material and the DNA. Particles fabricated with high weight percentages of PLGA-PLL/PLGA resulted in remarkably increased loading (>90%). Additionally, the release profile could be dictated by the quantity of PLGA-PLL incorporated into the particles. Particles incubated *in vitro* on COS-7 cells were able to transfect cells. These results demonstrated that DNA encapsulation and release were modulated by the method of fabrication.

Keywords

Plasmid DNA; PLGA; Poly-L-lysine; particle; COS-7

1. Introduction

Biodegradable particles have been widely explored as carriers for plasmid DNA over the last 10 years. In much of this work, poly(lactic-co-glycolic acid) (PLGA) has been used to fabricate micron and submicron sized particles due to its biocompatibility and history as a carrier for other therapeutic molecules. Incorporation of plasmid DNA into PLGA particles provides (1) protection of DNA from *in vivo* degradation, (2) a mechanism of controlled release, and (3) a transfection vehicle for mammalian cells. The most common application of this technology has been eliciting an immunological response through the delivery of plasmid DNA encoding an antigenic sequence specific to a given pathogen. For example, PLGA particles have been

*Corresponding author: W. Mark Saltzman, Department of Biomedical Engineering, MEC 414, Yale University, Prospect St, New Haven, CT 06511, Email: mark.saltzman@yale.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

fabricated with plasmid DNA encoding sequences from viral (HIV [1] and HBV [2,3]), bacterial (tuberculosis [4]), and parasitic (leishmania [5]) pathogens. Moreover, some of this work has translated into phase I and II clinical trials demonstrating efficacy in humans [6,7]. Beyond genetic vaccines, DNA delivery from particles has also been investigated for correction of genetic diseases [8] and tissue regeneration [9].

Incorporation of plasmid DNA with PLGA can be accomplished by either encapsulation within the particle [10,11] or adsorption to the surface of the particle [12]. Formation of particles is usually accomplished by a double emulsion technique, in which energy is introduced to the system typically by either sonication or homogenization. The amount of energy introduced by these agitation sources, along with the concentration of surfactant and/or presence of a co-solvent, dictate the size of the particles (generally 0.1–10 μm). When DNA is encapsulated or absorbed, these particles have biological function *in vitro* by transfection of mammalian cells and expression of reporter genes *in vivo* [8,12,13].

Traditionally, the release of molecules from PLGA is controlled by the composition of copolymer (ratio of lactic to glycolic acid) or the molecular weight of the polymer [14,15]. The size of the particle will also influence the release of a molecule from PLGA. Although there are approaches to control the release kinetics of macromolecules from PLGA, the degree of control is often limited. Another limitation of PLGA as a drug delivery carrier is the poor encapsulation efficiency of hydrophilic molecules such as DNA. This low retention is further compounded when submicron particles are fabricated. Both drug encapsulation and release kinetics are important considerations when developing drug delivery devices that will have specific interactions with mammalian cells. As a result, new techniques are needed to create submicron particles with highly controllable drug release and encapsulation characteristics.

This study explored strategies to fabricate submicron-sized PLGA particles for the encapsulation of plasmid DNA, which will (1) enhance DNA encapsulation within the particle, (2) provide a platform for high degree of control of DNA release, and (3) increase *in vitro* transfection of mammalian cells. The effect of agitation source and chemical conjugation of PLGA with the cationic polymer, poly-L-lysine, was investigated. These results provide new methods for highly efficient DNA encapsulation and controlled release.

2. Materials and methods

2.1 Synthesis of PLGA-poly-L-lysine

Conjugation of poly-L-lysine (PLL) to poly(D,L-lactide-co-glycolide (PLGA) was accomplished via coupling using dicyclohexyl carbodiimide (DCC) as previously established by Lavik et al. [16]. PLGA (3 g, 50:50 PLGA Acid End Group; i.v. ~ 0.67 dL/g; Absorbable Polymers: Pelham, AL) and poly(ϵ -carbobenzoxyl-L-lysine) in five molar excess (200 mg; 1,000–4,000 MW, Sigma-Aldrich, St Louis, MO) were dissolved in dimethylformamide (6 mL, DMF; Sigma-Aldrich) in a dry round-bottom flask under argon. DCC (58 mg; Sigma-Aldrich) and dimethylaminopyridine (0.31 mg, DMAP; Sigma-Aldrich) were dissolved in 2 mL DMF under argon, then added to the polymer solution and allowed to stir for ~ 48 hours. The reacted solution was diluted by the addition of chloroform and precipitated in methanol. The dried polymer was redissolved in chloroform, precipitated in ether, and then dried under vacuum for 24 hours. Unconjugated PLL was removed during precipitation and washes. Dried protected product was placed in a round bottom flask, purged with argon, and dissolved in 10 mL hydrogen bromide, 30% wt in acetic acid (Sigma-Aldrich) and allowed to stir for 90 minutes for deprotection. The polymer was precipitated in ether and washed until the product changed from a yellow to off-white appearance. The product was dissolved in chloroform and precipitated in ether. The polymer was vacuum dried for 24 hours to remove all trace ether.

Samples before and after deprotection were collected to confirm modification of the polymer and subsequent removal of protecting carbobenzoxy (CBZ) groups. The samples were dissolved in trifluoroethanol (TFE; Sigma-Aldrich) and evaluated from 200–350 nm using spectroscopy (Cary 50 Bio UV-Vis Spectrophotometer, Varian, Palo Alto, CA).

2.2 Plasmid DNA preparation

The CMV promoter was cloned upstream of the luciferase transgene in the pGL3 Basic (Promega, Madison, WI) vector. This plasmid was propagated in DH5 α bacteria cells and purified using the Qiagen's EndoFree Plasmid Giga Kit (Santa Clara, CA). The endotoxin level of plasmid preparation was < 0.1 EU/ml determined by the Limulus Amebocyte Lysate QCL-1000 Kit (Cambrex Biosciences, Walkersville, MD).

2.3 Particle fabrication

Particles were fabricated by a double emulsion water in oil in water (w/o/w) method. Briefly, 200 mg of polymer was dissolved overnight in 2 mL dichloromethane (10% wt/vol). 200 μ L of DNA (4 mg/mL in TE buffer) was added dropwise to the dissolved polymer under vortex and then sonicated on ice for 30 sec at 38% amplitude (Tekmar Sonic Disruptor TM300, Mason, Ohio). The primary emulsion was added dropwise to 4 mL of second aqueous solution (1% polyvinyl alcohol (PVA, Sigma-Aldrich), 10% sucrose) and then subjected to 30 sec sonication at 38% amplitude. The particle solution was added to 50 mL of 0.3% PVA and 10% sucrose, stirring for 3 hrs to allow for the evaporation of dichloromethane and hardening. The particles were collected by centrifugation at 8000 RPM and washed twice with sterile water. Lastly, the particles were suspended in 5 mL water, frozen, and freeze-dried. For particles fabricated with homogenization, 200 mg polymer was dissolved in dichloromethane in the presence of a co-solvent (TFE 1:5 ratio) resulting in a final volume of 2 mL. As with the sonication method, 200 μ L of DNA was added dropwise to the dissolved polymer under vortex and then agitated with a T25 Basic 25G homogenizer (IKA[®] Works INC, Wilmington, NC) at 24,000 RPM for 30 sec on ice. The primary emulsion was added dropwise to 4 mL of second aqueous solution (5% PVA, 10% sucrose) and then homogenized at 24,000 RPM for 30 sec. The subsequent hardening, washes, and freeze-drying steps were performed in the same way as in the protocol described above for the sonication-fabricated particles. Blank particles (i.e. without DNA) were prepared by using TE buffer in the first emulsion. Note that during particle fabrication, instruments and solutions were prepared to be endotoxin free and particles were prepared aseptically by heat sterilizing all equipment and filtering all solutions using 0.2 μ m membranes. Three batches of particles were made for this experiment with the exception of particles without DNA.

2.4 Particle characterization

2.4.1 DNA-loading—The amount of DNA unencapsulated during fabrication was determined by absorbance at 260 nm using UV-spectroscopy (Spectromax M5, Molecular Devices, Sunnyvale, CA) of the supernatant solution after the primary collection of particles. No DNA was detected in subsequent washes. The absorbance was recorded at 260 nm then multiplied by the extinction coefficient (50 μ g/mL) to determine the concentration of unencapsulated DNA:

$$\% \text{unencapsulated} = \left(\frac{\text{volume supernatant} \times \text{concentration}}{\text{initial mass DNA}} \right) \times 100\%$$

DNA loaded per mass particles was calculated as follows:

$$\text{loading} = \left(\frac{\text{initial mass DNA loaded} \left(1 - \frac{\% \text{loss}}{100\%} \right)}{200 \text{ mg particles}} \right)$$

2.4.2 Scanning electron microscopy (SEM)—Particles were characterized by SEM. Samples were mounted on carbon tape and sputter-coated with gold under vacuum in an argon atmosphere using a sputter current of 40 mA (Dynavac Mini Coater, Dynavac, USA). SEM analysis was carried out with a Philips XL30 SEM using a LaB electron gun with an accelerating voltage of 5kV.

2.4.3 Gel Electrophoresis—Plasmid DNA stability and topology were assessed by 1.0% agarose gel electrophoresis using TBE buffer (89 mM Tris, 89 mM Boric Acid, and 2 mM EDTA, pH~8.4) and SYBR Safe™ DNA gel staining dye (Molecular Probes, Eugene, OR). Encapsulated DNA was extracted from particles by dissolution in methylene chloride. After dissolution, TE buffer with 1% (wt/vol) Heparin sodium salt (Sigma) was added, vortexed, and rotated end-over-end overnight at room temperature. The mixture was centrifuged and the aqueous phase of the mixture was collected. Approximately 200 nanograms of DNA was run on a gel. Densometry analysis using Image J (NIH, Bethesda, MD) was used to determine the relative quantity of DNA in each isoform.

2.4.4 Particle sizing—The particle size was determined by analysis of three representative SEM images using Image J. The program determined the area of over 500 individual particles per sample and the diameter was back-calculated $Dia = 2(area/\pi)^{1/2}$. The average diameter and standard deviation was recorded for each formulation.

2.4.5 Zeta potential—The surface charge of the particles was evaluated using ZetaPlus (Brookhaven Instruments Corporation, Holtsville, NY). All samples were suspended in demineralized water and 15 measurements were taken per sample. The average surface charge and standard deviation was recorded for each formulation.

2.5 In vitro release of DNA

Approximately 2 mg of DNA particles were incubated in phosphate buffered saline pH ~7.4 at 37 °C on a shaker platform. Supernatant samples were taken at various timepoints for a period of 18 weeks. The quantity of DNA release in the supernatant was determined using the PicoGreen Assay (Molecular Probes, Eugene, OR). DNA quantity was calculated from a standard curve of known concentrations (5–2000 ng/mL).

2.6 In vitro bioactivity

COS-7 cells (ATCC, Manassas, VA) were maintained in α -MEM media supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 1% penicillin/streptomycin. Cells were plated in a 24 well plate at a concentration of 25×10^4 cells/well. The following day, medium was removed and replaced with medium containing particles (0.5 mL at a concentration of 1 mg/mL, 500 μ g per well); the particle-loaded medium was incubated with the cells for 24 hours. As controls, particles without DNA and 2 μ g plasmid DNA were also included in bioactivity evaluation. After incubation, the particles were removed, the cells were washed with 0.5 mL fresh medium, and then particle-free medium was added. Forty-eight hours later, media was removed and 250 μ L cell culture lysis reagent (Promega) was added. A sample of 20 μ L was added to 100 μ L luciferase assay reagent (Promega), which was read on a luminometer (Glomax 20/20, Promega) for a 10 sec integration time. Using the PicoGreen Assay, the quantity of cellular DNA was determined. Data was represented as relative luminescence units (RLU) per μ g cellular DNA.

2.7 Statistical Analysis

Particles for each experimental condition were fabricated in three unique batches. All data were taken in triplicate and reported as mean and standard deviation. For zeta potential and

bioactivity analyses, a paired Student's *t*-test of unequal variances with 95% confidence interval ($p < 0.05$) was done between blank control and experimental group.

3. Results

3.1 PLGA-PLL particles

Conjugation of PLL to PLGA was achieved through the coupling agent, DCC (Fig. 1A). PLL protected with CBZ groups was conjugated to PLGA confirmed by UV spectrometry via the absorption of CBZ peaks, which were visible at approximately 257 nm (Fig. 1B). After successful conjugation of PLL, a deprotection step was accomplished to remove the CBZ groups, leaving only PLL conjugated to PLGA (this was confirmed by the disappearance of the CBZ group peaks, Fig. 1B). These data were consistent with successful synthesis of the block copolymer of PLGA and PLL [16].

DNA-loaded particles were prepared using a double emulsion technique with either sonication or homogenization as agitation sources. A range of particles were fabricated at different percentages of PLGA-PLL/PLGA (wt/wt). The size of the particle depended primarily on the method of agitation; particles of average diameter and standard deviation of 270 ± 110 nm (sonication) and 450 ± 250 nm (homogenization) were produced (Table 1). For particles fabricated by sonication, size was not influenced by either the weight percentage of PLGA-PLL/PLGA incorporated or the presence of plasmid DNA. In general, both fabrication methods resulted in particles that followed a Gaussian size distribution (Fig. 2). Additionally, sonication prepared particles demonstrated a slightly more monodispersed distribution than those prepared by homogenization. All particles were spherical in morphology and relatively homogeneous in distribution (Fig. 2). No qualitative difference in morphology was observed due to the presence of PLGA-PLL (data not shown).

Incorporation of PLGA-PLL reduced the amount of plasmid DNA lost during fabrication and resulted in higher loading. For particles fabricated using sonication, unencapsulated DNA was reduced from more than 80% to less than 20% by incorporating greater than 25% PLGA-PLL/PLGA (wt/wt) (Table 1). When particles were fabricated using homogenization, unencapsulated DNA was completely eliminated by incorporating just 10% PLGA-PLL/PLGA (wt/wt) and resulted in a maximal loading of 4 μ g DNA per mg particle (Table 1). Gel electrophoresis demonstrated that both sonication and homogenization resulted in some disruption of supercoiled DNA into open circular form (Fig. 3). Semi-quantitative analysis by densometry revealed 30–50% (sonication) and 30–40% (homogenization) of the total DNA remained supercoiled (90% of distributed DNA was supercoiled). DNA extracted from particles was able to transfect cells at approximately the same efficiency between particle formulations, but relative to unmanipulated DNA, the efficiency was reduced by half (data not shown).

Zeta potential measurements were used to determine the surface charge on the particles prepared by sonication. The incorporation of PLGA-PLL reduced the overall negative surface charge in particles fabricated both with and without DNA (Fig. 4). The surface charge became positive for blank particles in which half or more PLGA-PLL/PLGA (% wt/wt) was incorporated, and only in these particles was there a statistical difference ($p < 0.05$) in surface charge between particles without and with DNA (Fig. 4). Of the formulations evaluated, only 100% PLGA-PLL/PLGA (wt/wt) resulted in a positively charged surface when fabricated with DNA (Fig. 4).

3.2 Controlled release of DNA from particles

Particles were suspended in PBS and incubated at 37 °C to determine the kinetics of DNA release. When different weight percentages of PLGA-PLL/PLGA (5%, 10%, 25%, 50%, and 100%) were incorporated into particles using sonication, a substantial increase in plasmid DNA release during the short-term (0–1 week) and long-term (1–18 weeks) was observed compared to pure PLGA particles (Figs. 5A, 5B). Increasing the percentage of PLGA-PLL/PLGA (wt/wt) in the particles reduced the burst release (i.e. release during the first day), which was observed when using sonication as an energy source (Fig. 5A). Over the course of the next 18 weeks, particles fabricated with high weight percentages of PLGA-PLL/PLGA (> 10%) demonstrated continued release and a secondary release of DNA between 7 to 8 weeks (Fig. 5B). PLGA-PLL/PLGA particles (10%, wt/wt) fabricated with homogenization resulted in a larger quantity of DNA released than pure PLGA particles fabricated by the same method (Fig. 5C). In contrast to particles prepared by sonication, which exhibited a burst release, particles prepared with homogenization yield a more linear release profile over 18 weeks (Fig. 5D).

3.3 In vitro bioactivity

The utility of these particles as a vehicle for transfection was evaluated using COS cells. Transfection efficacy for all particle preparations was determined by quantifying the expression of luciferase encoded on the plasmid. At higher weight percentages of PLGA-PLL/PLGA ($\geq 10\%$) in sonication fabricated particles, levels of luciferase expression were statistically greater than blank particles (Fig. 6). Particles with lower percentages of PLGA-PLL/PLGA (< 10%, wt/wt) exhibited no significant expression. Particles fabricated by homogenization also demonstrated no luciferase expression (data not shown). DNA released from particles between days 1 and 3 was correlated with bioactivity of sonication fabricated particles when incubated with COS cells (Fig. 7). DNA released in this time period (> 200 ng) corresponded to greater luciferase expression (or COS transfection).

4. Discussion

This study explored strategies for increasing the encapsulation efficiency, modulating the release kinetics, and improving the transfection ability of DNA loaded into PLGA particles. Two fabrication modifications were utilized to accomplish these goals: (1) chemical conjugation of poly-L-lysine to the termini of PLGA polymer to provide more favorable interactions between the polymer and DNA, and (2) the method of agitation (sonication or homogenization) in the fabrication process.

One of the benefits of sonication as an agitation source is the ability to fabricate particles that are both relatively small (submicron) and uniform. Unfortunately, sonication usually results in considerable DNA loss during encapsulation (> 80%, Table 1) and a substantial burst release within the first 24 hours. These unfavorable characteristics were overcome by the incorporation of PLGA-PLL into particles during fabrication. Blending five weight percent of PLGA-PLL/PLGA polymer resulted in a substantial reduction in plasmid loss. As more PLGA-PLL was incorporated into the particles, a corresponding reduction in DNA loss during encapsulation was observed. In addition, the magnitude of the initial burst release of DNA within the first 24 hours from the particles was controlled by addition of PLGA-PLL. While lower blends of PLGA-PLL/PLGA (5% and 10%, wt/wt) maintained a burst similar to PLGA alone (>40% within the first 24 hrs), higher blends of PLGA-PLL/PLGA (25%, 50%, and 100%; wt/wt) drastically reduced the burst release ($33 \pm 4\%$, $22 \pm 9\%$, and $11 \pm 4\%$ respectively). It is hypothesized that the diffusion of DNA from the emulsified droplets during particle hardening is slowed by electrostatic interactions with positively charged PLL. At eight weeks of incubation, a second release was observed. This was most likely due to the degradation of polymer by hydrolysis. The presence of primary amines in PLGA-PLL may also accelerate

hydrolysis, and subsequently, DNA release in particles. This may explain the increase in release rates observed in particles as the blend of PLGA-PLL increases from 25% to 100% despite similar DNA loadings. Both the increase in loading and the delay in release kinetics correlated with the surface charge of the particles: particles became more positively charged as increasing amounts of PLGA-PLL were incorporated. Interestingly, particles incorporating as little as 25% PLGA-PLL/PLGA (wt/wt) achieved close to maximal DNA loading.

Homogenization is another commonly used method of agitation for fabricating DNA-loaded particles [8,17]. In this approach, emulsions were created through high speed mixing (24,000 RPM) of DNA/polymer in the presence of a surfactant. In comparison with sonication, producing submicron particles by homogenization required a higher concentration of surfactant (5% vs. 1%) and the use of a co-solvent. Even with these changes, on average, particles fabricated with homogenization were slightly larger than those produced by sonication (~450 nm vs. ~280 nm). One advantage of this technique was that considerably less DNA was left unencapsulated during fabrication (~50%). The differences in unencapsulated DNA between the two methods may be explained by (1) the slight increase in particle size (which yields better loading), and (2) the manner in which DNA associates within the emulsion during particle formation by the specific energy sources. Another possible contributing factor in enhanced DNA encapsulation efficiency and more thorough distribution in particles was the presence of the co-solvent, TFE, which resulted in faster hardening and hindered DNA diffusion out of the particle during fabrication by homogenization. It was also demonstrated that when a relatively small amount of PLGA-PLL/PLGA (10%, wt/wt) was incorporated into the particles fabricated by homogenization, the loss of DNA during loading was completely eliminated. Thus, independent of agitation method, incorporation of PLGA-PLL within the formulation results in a higher loading of plasmid DNA within the particles. Both agitation methods resulted in 30–50% of total DNA in the supercoiled isoform after encapsulation.

Particles fabricated by homogenization had slower, more linear, DNA-release profiles than particles fabricated by sonication. There are two possible reasons behind the differences in the release profiles. The first is differences in area-to-volume ratio. A more speculative explanation may be differences in the distribution of DNA within the particle. If homogenization indeed results in a more uniform distribution of DNA throughout the particles, then it would take longer for the dispersed molecules to diffuse out of the particles. Particles with 10% PLGA-PLL/PLGA (wt/wt) incorporated demonstrated the same relative DNA-release profile as particles without PLGA-PLL. Increases above 10% were not explored because maximum loading was achieved with this fabrication method.

The functionality of particles were examined by transfecting COS cells in tissue culture. In these studies it was observed that the particles fabricated using sonication were able to transfect mammalian cells; the extent of transfection depended on the blend of PLGA-PLL/PLGA (wt/wt) incorporated into the particles. Transfection occurred even with changes in DNA isoform during fabrication. Further studies are needed to address the reliability of DNA incorporated in particles. The differences in transfection efficiency between the sonicated formulations can be explained by the release kinetics and cell culture model. The particles were incubated on the cells for 24 hours, removed, rinsed, and replaced with new medium. Two days later, luciferase expression was evaluated; transgene expression correlated with DNA released between 1–3 days (Fig. 7). In the formulations where less PLGA-PLL was incorporated (which resulted in a larger burst), the majority of DNA may have been released prior to internalization into the cells (< 1 day). Poor transfection with lower weight percentages of PLGA-PLL/PLGA may also be a result of the magnitude of negative charge on the surface of the particles. Figure 4 illustrates that 25% PLGA-PLL/PLGA (wt/wt) and greater a reduction in negative surface charge, which results in much more favorable electrostatic interactions with the cell membrane, facilitating internalization into the cells. Cationic charge on the surface of microparticles has

demonstrated increased uptake of particles in both monocytes and dendritic cells [18]. Particles fabricated with homogenization did not transfect cells under the conditions investigated. One explanation for their failure to transfect cells may be that an inadequate amount of DNA was delivered to the cell to generate transfection after three days. In addition, barriers of internalization such as surface charge and particle size may also have been contributing factors.

The use of cationic polymers to condense plasmid DNA for transfection of mammalian cells has been well studied. More recently, this interaction has been explored to enhance the transfection ability of biodegradable particles. For example, Capan et al. have encapsulated DNA previously complexed with PLL into PLGA microparticles and demonstrated the ability to control release of these complexes by using different molecular weight polymers or changing the surfactant concentration [19,20]. The cationic surfactant cetyltrimethylammonium bromide (CTAB) was shown effective for absorbing DNA onto the surface microparticles [12,21]. In this strategy, the concentration of CTAB used during fabrication dictates both the loading and the amount of DNA released within the first 24 hours. Similarly, PLL has been able to change the surface charge when used as an emulsifier in the fabrication PLGA particles [22,23]. Plasmid DNA absorbed on PLL coated polystyrene nanoparticles has been shown to transfect dendritic cells [24]. Others have utilized surface adsorption of DNA by conjugating another common transfection cationic polymer, polyethyleneimine (PEI), to PLGA [13]. In these studies, the release profile was dictated by the molecular weight of PEI and whether it was branched or linear in structure [25]. A concern with coating the surface of particles with cationic polymers is cellular toxicity. At high coating concentrations of CTAB or PEI, membrane toxicity was observed in cells [26].

The grafting of poly(L-lysine) to PLGA has been previously reported as a gene delivery carrier [27]. In this approach, the modified polymer complexed with plasmid DNA was used to form nano-sized micelles. The studies presented in this paper differ in that the particles formed were a depot from which DNA is released over a short and long time scale.

Utilizing the same electrostatic interactions, but in a different molecular format, this study presents a new platform for controlled release of DNA. Unlike other strategies, which are limited by the manufacturer's selection of molecular weight polymers, controlling the magnitude of the burst release was accomplished relatively easily, by simply changing the amount of PLGA-PLL incorporated into the particles. An additional benefit of this method is that incorporation of PLGA-PLL dramatically improves the loading efficiency of plasmid DNA. These particles may prove valuable in providing a tunable range of release profiles to explore that may be specific for a given application. For example, a robust burst release may be necessary when trying to initiate an immune response, whereas slower, more persistent release may be desirable for a plasmid encoding a growth factor for tissue regeneration. Alternatively, the superposition of both release profiles provides different therapeutic delivery ranges which may ultimately lead to the desired response. Clinically, it is envisioned that these particles would be administered orally, intranasally, or injected at a local tissue site. To date, the modified polymer has not been tested with other hydrophilic anionic agents for delivery, but it is anticipated that such agents will exhibit similar characteristics (enhanced loading and control over release profile) to what was observed with DNA.

5. Conclusion

This study explored unique manipulations to control the encapsulation and subsequent release of plasmid DNA from submicron particles. The emulsifying energy source (homogenization or sonication) and the conjugation of poly-L-lysine to PLGA both affected the encapsulation efficiency and the release profile (linear vs burst) from particles. Functionally, it was demonstrated that particles fabricated with a high percentage (>50%) of PLGA-PLL resulted

in transfection of COS-7 cells. Further studies are needed to demonstrate practical benefit of these particles *in vivo*.

Acknowledgements

The authors thank James Bertran and Erin Lavik (Yale University, New Haven, CT) for their invaluable discussions about the synthesis of PLGA-PLL and provision of the PLGA-PLL conjugation schematic (Fig. 1A). The pCMV-Luc plasmid was generously provided by Dr. Michael Barry (Mayo Clinic, Rochester, MN). This work was supported by a grant from the National Institutes of Health (EB000487) (WMS).

References

1. Denis-Mize KS, Dupuis M, Singh M, Woo C, Ugozzoli M, O'Hagan DT, Donnelly JJ, Ott G, McDonald DM. Mechanisms of increased immunogenicity for DNA-based vaccines adsorbed onto cationic microparticles. *Cell Immunol* 2003;225(1):12–20. [PubMed: 14643300]
2. He X, Jiang L, Wang F, Xiao Z, Li J, Liu LS, Li D, Ren D, Jin X, Li K, He Y, Shi K, Guo Y, Zhang Y, Sun S. Augmented humoral and cellular immune responses to hepatitis B DNA vaccine adsorbed onto cationic microparticles. *J Control Release* 2005;107(2):357–372. [PubMed: 16099068]
3. He XW, Wang F, Jiang L, Li J, Liu SK, Xiao ZY, Jin XQ, Zhang YN, He Y, Li K, Guo YJ, Sun SH. Induction of mucosal and systemic immune response by single-dose oral immunization with biodegradable microparticles containing DNA encoding HBsAg. *J Gen Virol* 2005;86:601–610. [PubMed: 15722520]
4. Lima KM, Santos SA, Lima VMF, Coelho-Castelo AAM, Rodrigues JM, Silva CL. Single dose of a vaccine based on DNA encoding mycobacterial hsp65 protein plus TDM-loaded PLGA microspheres protects mice against a virulent strain of *Mycobacterium tuberculosis*. *Gene Ther* 2003;10(8):678–685. [PubMed: 12692596]
5. Coelho EAF, Tavares CAP, Lima KD, Silva CL, Rodrigues JM, Fernandes AP. *Mycobacterium hsp65* DNA entrapped into TDM-loaded PLGA microspheres induces protection in mice against *Leishmania* (*Leishmania*) major infection. *Parasitol Res* 2006;98(6):568–575. [PubMed: 16432754]
6. Garcia F, Petry KU, Muderspach L, Gold MA, Braly P, Crum CP, Magill M, Silverman M, Urban RG, Hedley ML, Beach KJ. ZYC101a for treatment of high-grade cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet Gynecol* 2004;103(2):317–326. [PubMed: 14754702]
7. Klencke B, Matijevic M, Urban RG, Lathey JL, Hedley ML, Berry M, Thatcher J, Weinberg V, Wilson J, Darragh T, Jay N, Da Costa M, Palefsky JM. Encapsulated plasmid DNA treatment for human papillomavirus 16-associated anal dysplasia: A phase I study of ZYC101. *Clin Cancer Res* 2002;8(5):1028–1037. [PubMed: 12006515]
8. Stern M, Ulrich K, Geddes DM, Alton EFWF. Poly (D, L-lactide-co-glycolide)/DNA microspheres to facilitate prolonged transgene expression in airway epithelium *in vitro*, *ex vivo* and *in vivo*. *Gene Ther* 2003;10(16):1282–1288. [PubMed: 12883524]
9. Yi F, Wu H, Jia GL. Formulation and characterization of poly (D,L-lactide-co-glycolide) nanoparticle containing vascular endothelial growth factor for gene delivery. *J Clin Pharm Ther* 2006;31(1):43–48. [PubMed: 16476119]
10. Hedley ML, Curley J, Urban R. Microspheres containing plasmid-encoded antigens elicit cytotoxic T-cell responses. *Nat Med* 1998;4(3):365–368. [PubMed: 9500615]
11. Luo D, Woodrow-Mumford K, Belcheva N, Saltzman WM. Controlled DNA delivery systems. *Pharm Res* 1999;16(8):1300–1308. [PubMed: 10468035]
12. Singh M, Briones M, Ott G, O'Hagan D. Cationic microparticles: A potent delivery system for DNA vaccines. *Proc Natl Acad Sci U S A* 2000;97(2):811–816. [PubMed: 10639162]
13. Kasturi SP, Sachaphibulkij K, Roy K. Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. *Biomaterials* 2005;26(32):6375–6385. [PubMed: 15913771]
14. Tinsley-Bown AM, Fretwell R, Dowsett AB, Davis SL, Farrar GH. Formulation of poly(D,L-lactic-co-glycolic acid) microparticles for rapid plasmid DNA delivery. *J Controlled Release* 2000;66(2–3):229–241.

15. Wang DQ, Robinson DR, Kwon GS, Samuel J. Encapsulation of plasmid DNA in biodegradable poly (D,L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. *J Controlled Release* 1999;57(1):9–18.
16. Lavik EB, Hrkach JS, Lotan N, Nazarov R, Langer R. A simple synthetic route to the formation of a block copolymer of poly(lactic-co-glycolic acid) and polylysine for the fabrication of functionalized, degradable structures for biomedical applications. *J Biomed Mater Res* 2001;58(3):291–294. [PubMed: 11319743]
17. Barman SP, Lunsford L, Chambers P, Hedley ML. Two methods for quantifying DNA extracted from poly(lactide-co-glycolide) microspheres. *J Controlled Release* 2000;69(3):337–344.
18. Wischke C, Borchert HH, Zimmermann J, Siebenbrodt I, Lorenzen DR. Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. *J Control Release* 2006;114(3):359–368. [PubMed: 16889866]
19. Capan Y, Woo BH, Gebrekidan S, Ahmed S, DeLuca PP. Influence of formulation parameters on the characteristics of poly(D, L-lactide-co-glycolide) microspheres containing poly(L-lysine) complexed plasmid DNA. *J Control Release* 1999;60(2–3):279–286. [PubMed: 10425333]
20. Capan Y, Woo BH, Gebrekidan S, Ahmed S, DeLuca PP. Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly(L-lysine) complexed plasmid DNA. *Pharm Res* 1999;16(4):509–513. [PubMed: 10227704]
21. Singh M, Ugozzoli M, Briones M, Kazzaz J, Soenawan E, O'Hagan DT. The effect of CTAB concentration in cationic PLG microparticles on DNA adsorption and in vivo performance. *Pharm Res* 2003;20(2):247–251. [PubMed: 12636163]
22. Cui C, Stevens VC, Schwendeman SP. Injectable polymer microspheres enhance immunogenicity of a contraceptive peptide vaccine. *Vaccine* 2007;25(3):500–509. [PubMed: 16996662]
23. Cui CJ, Schwendeman SP. Surface entrapment of polylysine in biodegradable poly(DL-lactide-co-glycolide) microparticles. *Macromolecules* 2001;34(24):8426–8433.
24. Minigo G, Scholzen A, Tang CK, Hanley JC, Kalkanidis M, Pietersz GA, Apostolopoulos V, Plebanski M. Poly-L-lysine-coated nanoparticles: a potent delivery system to enhance DNA vaccine efficacy. *Vaccine* 2007;25(7):1316–1327. [PubMed: 17052812]
25. Kasturi SP, Qin H, Thomson KS, El-Bereir S, Cha SC, Neelapu S, Kwak LW, Roy K. Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. *J Controlled Release* 2006;113(3):261–270.
26. Oster CG, Kim N, Grode L, Barbu-Tudoran L, Schaper AK, Kaufmann SH, Kissel T. Cationic microparticles consisting of poly(lactide-co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination. *J Control Release* 2005;104(2):359–377. [PubMed: 15907586]
27. Jeong JH, Park TG. Poly(L-lysine)-g-poly(D,L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. *J Controlled Release* 2002;82(1):159–166.

A

PLGA-PLL CONJUGATION

B

Fig. 1. Poly-L-lysine (PLL) was conjugated to PLGA. (A) In this synthesis, dicyclohexyl carbodiimide (DCC) was used as a coupling agent, which activated the carboxylic acid end group on the PLGA for conjugation to the free amine on PLL. (B) UV-Vis spectra shows PLGA conjugated to CBZ protected PLL (solid line) and subsequent deprotection of lysine by removal of CBZ group (dotted line). The peaks around 257 nm correspond to the CBZ protecting group on PLL.

Fig. 2. Size distribution and scanning electron micrographs (SEM) of particles fabricated by (A) sonication and (B) homogenization. Bar represents 1 μm .

Fig. 3.

DNA extracted from particles. Lanes: (1) DNA ladder (1–10 kb); (2) unincorporated DNA; (3,4) DNA extracted from particles fabricated by homogenization; and (5–10) DNA extracted from particles fabricated by sonication. DNA extracted from particles containing (3,5) 0%; (6) 5%; (4,7) 10%; (8) 25%; (9) 50%; and (10) 100% PLGA-PLL/PLGA (wt/wt). Supercoiled (sc) and open circular (oc) are indicated.

Fig. 4. Surface charge (or zeta potential) of particles prepared with different weight percentages of PLGA-PLL/PLGA. Particles were prepared without (white bars) or with (dark bars) plasmid DNA. Particles fabricated with 50% and 100% PLGA-PLL/PLGA (wt/wt) demonstrated a statistically significant difference (* $p < 0.05$) in surface charge between particles with and without plasmid DNA. Error bars indicate standard deviation ($\pm$ SD) for $n = 3$.

Fig. 5.

Controlled release of plasmid DNA from particles. (A) First seven days of release of DNA (ng) from particles containing different weight percentages of PLGA-PLL/PLGA, 0% (□), 5% (▲), 10% (■), 25% (◆), 50% (▼), and 100% (✱) prepared by sonication. Errors bars indicate standard deviation (n=3). (B) One to eighteen weeks release of DNA (ng) from particles containing different weight percentages of PLGA-PLL/PLGA prepared by sonication. (C) Release of DNA (ng) from particles without (○) or with (●) 10% PLGA-PLL/PLGA (wt/wt) prepared by homogenization. (D) Percentage of DNA released from particles without (□ ○) or

with (■ ○) 10% PLGA-PLL prepared by sonication (□ ■) or homogenization (○ ●). Error bars represent standard deviation.

Fig. 6.

In vitro transfection of COS cells. Luciferase expression in cells after 24-hour incubation with sonication fabricated particles. Luciferase activity represented in relative luminescence units (RLU). As controls, particles fabricated without DNA and 2 μ g plasmid DNA were included. A statistically significant increase in luciferase activity was observed in particles containing 10% (** $p < 0.1$), 25% (** $p < 0.1$), 50% (* $p < 0.05$), and 100% (* $p < 0.05$) PLGA-PLL/PLGA (wt/wt). Data normalized to cellular DNA. Bars represent mean $\pm$ SD for $n = 3$.

Fig. 7. Quantity of DNA released from sonication fabricated particles during 1–3 days of incubation and luciferase activity of COS cells transfected with particles. The percent PLGA-PLL incorporated into particles appears next to the data. Bars represent mean $\pm$ SD for $n=3$.

Table 1
Properties of particles prepared by sonication (S) and homogenization (H)

PLGA-PLL/ PLGA (% wt/wt)	Fabrication Method	w/o DNA		w/DNA		Loading $\pm$ SD (μ g/mg)
		Size $\pm$ SD (nm)	Size $\pm$ SD (nm)	% Unencapsulated $\pm$ SD	Size $\pm$ SD (nm)	
0	S	280 $\pm$ 110	270 $\pm$ 100	82 $\pm$ 6		0.7
5	S	280 $\pm$ 110	260 $\pm$ 100	34 $\pm$ 4		2.6
10	S	290 $\pm$ 110	280 $\pm$ 100	20 $\pm$ 8		3.2
25	S	270 $\pm$ 110	250 $\pm$ 110	6 $\pm$ 3		3.8
50	S	300 $\pm$ 120	270 $\pm$ 110	6 $\pm$ 2		3.8
100	S	290 $\pm$ 110	280 $\pm$ 110	11 $\pm$ 8		3.6
0	H	-	440 $\pm$ 220	51 $\pm$ 20		2.0
10	H	-	470 $\pm$ 270	0 $\pm$ 0		4.0