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Preparation, characterization, cytotoxicity and transfection efficiency of poly (DL-lactide-co-glycolide) and poly (DL-lactic acid) cationic nanoparticles for controlled delivery of plasmid DNA

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Abstract

The objective of this study was to investigate the effect of formulation parameters (i.e., polymer molecular weight and homogenization speed) on various physicochemical and biological properties of cationic nanoparticles. Cationic nanoparticles were prepared using different molecular weights of poly (DL-lactide-co-glycolide) (PLGA) and poly (DL-lactic acid) (PLA) by double emulsion solvent evaporation at two different homogenization speeds, and were characterized in terms of size, surface charge, morphology, loading efficiency, plasmid release, plasmid integrity, cytotoxicity, and transfection efficiency. Cationic surfactant, cetyltrimethylammonium bromide (CTAB), was used to provide positive charge on the surface of nanoparticles. Reporter plasmid gWIZTM Beta-gal was loaded on the surface of nanoparticles by incubation. Use of higher homogenization speed and lower molecular weight polymer led to a decrease in mean particle size, increase in zeta potential, increase in plasmid loading efficiency, and a decrease in burst release. The nanoparticles displayed good morphology as evident from scanning electron micrographs. In vitro cytotoxicity study by MTT assay showed a low toxicity. Structural integrity of the pDNA released from nanoparticles was maintained. Transfecting Human Embryonic Kidney (HEK293) cells with nanoparticles prepared from low molecular weight PLGA and PLA resulted in an increased expression of beta-galactosidase as compared to those prepared from high molecular weight polymer. Our results demonstrate that the PLGA and PLA cationic nanoparticles can be used to achieve prolonged release of pDNA, and the plasmid release rate and transfection efficiency are dependent on the formulation variables.

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Keywords

Poly (DL-lactide-co-glycolide) (PLGA); Poly (DL-lactic acid) (PLA); Cationic nanoparticles; Plasmid DNA (pDNA); Cytotoxicity; Transfection efficiency

1. Introduction

The turn of the century has seen enormous progress being made in the field of gene therapy. This strategy has been explored in the treatment of gene related disorders such as cystic fibrosis (Alton and Geddes, 1995; Griesenbach et al., 2004), severe combined immunodeficiency syndrome (Otsu and Candotti, 2002; Cavazzana-Calvo et al., 2005), cancer (Zwiebel et al., 1993; Kashani-Sabet, 2004), cardiovascular diseases (Shah et al., 2005), and AIDS (Strayer et al., 2005; Vanniasinkam and Ertl, 2005). Several thousand patients have been involved in clinical trials going on all over the world with majority focusing on cancer (66%), followed by monogenic diseases (8.7%), and vascular diseases (8.7%) (<http://www.wiley.co.uk/genetherapy/clinical/>). The basic concept of gene therapy is that human diseases may be treated by the transfer of genetic material into specific cells of a patient in order to correct or supplement the defective gene. Though gene therapy holds great promise for the achievement of this task, the transfer of genetic material into higher organisms still remains an enormous technical challenge (Wiethoff and Middaugh, 2003).

In order to enhance gene therapy, specialized design features are required for delivery vector to overcome extracellular and intracellular barriers, and ensure efficient DNA delivery to the nucleus (Wiethoff and Middaugh, 2003; Lechardeur and Lukacs, 2002). The vectors used for gene delivery are broadly classified as viral and non-viral vectors. Viral vectors account for about 75% of the clinical protocols currently in operation (<http://www.wiley.co.uk/genetherapy/clinical/>). However, there are some serious concerns associated with viral vectors such as immunogenicity, insertional mutations, and potential pathogenicity. Non-viral methods have received considerable attention lately because of their low immunogenic potential, ease of manufacture and safety. Most common non-viral approaches include use of neutral or cationic polymers and liposomes.

Polymeric systems for gene delivery have attracted much attention due to their favorable physico-chemical characteristics that make them suitable candidates for gene delivery application. Several polymers such as polyethyleneimine (PEI), polymethacrylates, poly (DL-lactide-co-glycolide) (PLGA) and poly (DL-lactic acid) (PLA) have been extensively investigated for gene delivery (Kasturi et al., 2005; Jones et al., 1997; Kim et al., 2005). These biodegradable polymers undergo bulk hydrolysis thereby providing sustained delivery of the therapeutic agent depending on the polymer molecular weight and copolymer composition (Ramchandani et al., 1997). The degradation products, lactic acid and glycolic acid, are removed from the body through citric acid cycle (Shive and Anderson, 1997). PLGA and PLA particles are matrix-type systems prepared by emulsification followed by evaporation of the organic phase. They have been used for controlled delivery in several studies and have shown good efficiency and biocompatibility both in vitro and in vivo (Shive and Anderson, 1997; Kang and Singh, 2005).

Two particles based gene delivery strategies have been pursued by researchers; encapsulation of DNA into the polymer matrix, or loading of anionic DNA on the surface of cationic particles. Encapsulation strategy has been conventionally used for delivery of DNA (Jones et al., 1997; Wang et al., 1999). Particles encapsulating DNA have showed good efficacy in animal models and have already completed phase II clinical trials. Still, there exist some fundamental limitations to this technique such as; creation of low pH microenvironment during degradation of polymer, low encapsulation efficiency, inability to escape phagolysosomes and low bioavailability. In addition, DNA is subjected to chemical, thermal and mechanical stresses during the production of particles leading to extensive degradation (Ando et al., 1999).

Surface loading of DNA on particles is a relatively newer approach (Singh et al., 2000; Esposito et al., 1999). Plasmid DNA is a polyanionic molecule which can be loaded on a polycationic surface due to ionic interaction. A cationic surfactant or polymer or a combination is used for preparation of cationic particles which, apart from facilitating plasmid loading, also, by virtue of its positive charge, helps in attachment of the particles on cell surface. This approach has the advantage of improved loading and increased bioavailability. PLGA particles functionalized using cetyltrimethylammonium bromide (CTAB), in particular, have aroused much interest due to biodegradability and proven safety profile of the polymer (Denis-Mize et al., 2000; Singh et al., 2000; Oster et al., 2005; Wischke et al., 2006). CTAB, which is a cationic surfactant, is incorporated on the surface of particles during emulsification, thereby providing a highly positive zeta potential. PLGA/CTAB particles showed high loading efficiency and were able to release plasmid DNA maintaining a predominantly supercoiled conformation (Singh et al., 2000). PLGA/CTAB particles have been successfully used for delivering DNA vaccine for immunization in mice (Luo et al., 2003; O'Hagan et al., 2004; He et al., 2005).

Effect of modulating different formulation variables on stability and in vitro release of nucleic acids encapsulated into PLGA/PLA microparticles and nanoparticles has been extensively studied (Walter et al., 1999; Diez and Tros de Ilarduya, 2006). A multitude of factors such as size of pDNA, polymer molecular weight, polymer amphiphilicity, size and nature of the entrapped molecule, nature of the solvent, formulation conditions, nature and concentration of surfactant are known to affect physicochemical characteristics of particles. Factors affecting properties of particles with a surface loaded molecule need to be thoroughly investigated.

In this study, we attempted to evaluate the effect of polymer molecular weight and homogenization speed on the characteristics of PLGA/CTAB and PLA/CTAB nanoparticles. Cationic nanoparticles were prepared by double emulsion/ solvent evaporation using CTAB in external aqueous phase to provide positive charge on the surface of particles. Cationic nanoparticles were evaluated for physicochemical characteristics such as size, morphology, surface charge and plasmid loading efficiency. The plasmid release rate of nanoparticles was studied in vitro and the conformational stability of released plasmid was determined. In vitro cytotoxicity, and transfection efficiency (both fresh and after storage) of nanoparticles were also determined.

2. Materials and methods

2.1. Materials

The plasmid encoding beta-galactosidase, gWiz™β-gal was obtained from Aldevron LLC (Fargo, ND, USA). Poly (DL-lactic acid) (PLA) (Inherent visc. 0.2dL/g, MW ≈ 10,000) was purchased from Polysciences Inc. (Warrington, PA, USA), polymers Poly (DL-lactide-co-glycolide) (PLGA) (inherent visc. 0.44dL/g, MW ≈ 50,000 and 0.63 dL/g, MW ≈ 100,000) and Poly (DL-lactic acid) (PLA) (inherent visc. 0.67 dL/g, MW ≈ 106,000) were purchased from Birmingham Polymers, Inc. (Birmingham, AL, USA). Cetyltrimethylammonium bromide (CTAB) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Human embryonic kidney (HEK293) cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Beta-galactosidase enzyme assay system with reporter lysis buffer was purchased from Promega corp. (Madison, WI, USA). Micro BCA™ protein assay reagent kit was purchased from Pierce Biotechnology (Rockford, IL, USA). De-ionized water was used to prepare all solutions and buffers.

2.2. Preparation of cationic nanoparticles

Nanoparticles were prepared using double emulsion/solvent evaporation technique. Briefly, 250 mg polymer was dissolved in 10 ml dichloromethane (DCM) and 1 ml phosphate buffered saline (PBS) was dispersed into it by sonication at 80W for 1 min using an ultrasonic homogenizer (Model 150 V/T, Biologics Inc., Manassas, VA, USA) to form a primary w/o emulsion. This emulsion was then added to 50 ml aqueous solution of cetyltrimethylammonium bromide (CTAB) (0.5% w/v) and homogenized for 12 h at predetermined homogenization speeds of 3000 or 5000 rpm using a homogenizer (Model L4RT, Silverson, UK) leading to the formation of secondary w/o/w emulsion and subsequent evaporation of the organic phase. The resultant nanoparticles were separated by centrifugation at 10,000 rpm. The particles were washed twice by re-suspension in distilled water (5 ml) followed by centrifugation at 10,000 rpm to remove excess surfactant. Finally, the particles were lyophilized for 24 h.

2.3. Particle size and zeta potential

Average particle diameters were measured via dynamic light scattering technique on a PSS/NICOMP 380 DLS particle sizing system (Santa Barbara, CA, USA). The samples were diluted in distilled water (pH 6.8) using autodilution mode. Six sub-runs were used for each measurement.

The zeta potential was measured on Zetasizer nano version 4.0 (Malvern Instruments, U.K.). A concentration of 0.1 –0.5% w/w was chosen for the zeta potential measurements. The measurements were done in distilled water (pH 6.8) using disposable zeta cells (DTS 1060) using the general purpose protocol at 27°C. A manual duration of about 30 sub-runs was used for each measurement. The mean zeta potential was determined using phase analysis light scattering technique.

2.4. Scanning electron microscopy

Particle morphology of selected batches was determined by scanning electron microscopy (SEM). The samples were sprinkled onto carbon tape attached to aluminum mounts followed by coating with gold using a Technics Hummer II sputter coater. Images were obtained using a JEOL JSM-6300 Scanning Electron Microscope.

2.5. Plasmid DNA loading on nanoparticles

Loading of reporter plasmid gWiz™ Beta-gal was performed on the surface of nanoparticles. The particles were suspended in a solution of pDNA (0.5 mg/ml) in PBS at a theoretical loading of 1% w/w at 4°C for 6 h on a shaking platform followed by centrifugation at 10,000 rpm. Loaded nanoparticles were washed twice with PBS and the supernatants were collected and analyzed at 260 nm to determine plasmid loading efficiency. The nanoparticles were finally resuspended in distilled water, lyophilized and stored at -20°C.

2.6. In vitro plasmid release

Plasmid release from cationic nanoparticles was studied in phosphate buffered saline (PBS) (pH 7.4). Ten milligrams of pDNA loaded nanoparticles were incubated in 1 ml PBS at 37°C in a reciprocal shaking water bath. At fixed time intervals, samples were removed by centrifugation and the amount of free DNA was determined in the supernatant at 260nm by UV spectrophotometer. The particles were re-suspended in fresh media.

2.7. Structural integrity of plasmid DNA

Structural integrity of pDNA was evaluated both immediately after loading and at different time points in the released samples by agarose gel electrophoresis. To determine plasmid stability immediately after surface loading, 10 mg loaded nanoparticles were dissolved in 2 ml chloroform and the plasmid was extracted by adding 2 ml PBS followed by agitation. DNA samples recovered from nanoparticles by extraction and the samples released at different time points (i.e. 0.5, 1, 3, 7, and 14 days) along with control untreated pDNA were applied to 0.7% agarose gel in 1X Tris-Acetate EDTA (TAE) buffer. Band separation for supercoiled and open circular pDNA was observed after gel electrophoresis at 5V/cm² for 45 min.

2.8. Cytotoxicity evaluation by MTT assay

Cytotoxicity of selected formulations was determined by (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay in Human Embryonic Kidney (HEK293) cells (ATCC CRL-1573). This assay is based on the ability of living cells to reduce a water-soluble yellow dye, MTT, to a purple colored water-insoluble formazan product by mitochondrial enzyme succinate dehydrogenase. The cells were maintained in modified Minimum essential medium (Eagle) (EMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO₂ incubator at 37°C. Eight thousand cells were seeded per well in 96-well microtiter plates followed by incubation for 24 h. Nanoparticles at different concentrations, 2.5-50 µg per well in 100 µl media, were added to the wells. After 20 h incubation, 20 µl MTT solution (5 mg/ml in PBS, pH 7.4) was added to cells followed by further incubation

for 4 h. Thereafter, the media was removed and cells were rinsed with PBS. The formazan crystals formed were dissolved using dimethyl sulfoxide (DMSO) (100µl/well) and absorbance was read at 570 nm on a microplate reader. Cell viability was determined as a percentage of the negative control (untreated cells).

2.9. Expression of beta-galactosidase

Human Embryonic Kidney (HEK293) cells were plated in 24 well plates at a density of 2×10^5 cells/well in EMEM supplemented with 10% FBS and cultured for 24h. Effect of storage on transfection efficiency of nanoparticles was studied by comparing the expression of beta-galactosidase using freshly prepared nanoparticles with nanoparticles stored at -20°C for two weeks. Nanoparticles were added to cells at a pDNA equivalent dose of 500ng/well to the cells in 1 ml of complete cell culture medium followed by 48h incubation. Naked DNA was also added at equivalent dose. Following incubation, the media was removed; cells were washed with PBS, and lysed. Expression of beta-galactosidase was quantified by beta-galactosidase enzyme assay according to manufacturer's protocol. The total protein content of the samples was measured by Micro BCA™ protein assay (Smith et al., 1985).

3. Results and Discussion

3.1. Particle size and Zeta potential

Table 1 summarizes the results of physical characterization of nanoparticles. The mean particle size was found to depend on both polymer molecular weight and homogenization speed. Higher homogenization speed resulted in particles with a lower mean diameter as compared to lower homogenization speed irrespective of the polymer type or molecular weight. Higher homogenization speed leads to formation of smaller droplets due to increased shear during emulsification which may subsequently result in formation of smaller particles. Nanoparticles prepared with lower molecular weight PLGA or PLA had slightly lower mean size than particles prepared using higher molecular weight polymers. Increase in polymer molecular weight causes an increase in viscosity of the organic solvent phase which results in formation of larger particles. Similar results have been reported by other investigators on the influence of polymer molecular weight on particle size (Diez and Tros de Ilarduya, 2006). Polydispersity index (PDI) of formulations was between 0.26-0.43. Although studies have reported high polydispersity especially with multiple emulsion technique, such size distribution is not ideal particularly for in vivo application. Further attempts will be made to lower the polydispersity of the nanoparticles by modifying various formulation parameters.

Zeta potential of formulations prepared at higher homogenization speed was significantly higher ($p < 0.05$) as compared to those prepared at lower homogenization speed irrespective of the polymer used (Table 1). Such increase in zeta potential may be due to relatively higher amount of strongly bound CTAB on the surface of particles prepared at higher speed. Investigators have previously reported a higher amount of surface-associated stabilizer in submicron particles as compared to larger particles (Panyam et al., 2003). As an increase in the homogenization speed leads to formation of smaller particles, it may also result in

increased binding of CTAB on the surface causing an increase in the zeta potential. Particles prepared with lower molecular weight polymer had significantly higher ($p<0.05$) zeta potential as compared to higher molecular weight polymer irrespective of polymer type or homogenization speed. This may be due to more hydrophilic nature of low molecular weight polymers which may result in higher incorporation of hydrophilic CTAB on the oil-water interphase.

3.2. Scanning Electron Microscopy

SEM images (Fig. 1) revealed that all the nanoparticles formulations observed displayed good morphology with spherical structure. Nanoparticles had smooth and non-porous surface and were non-aggregated unlike one previous study that showed presence of highly aggregated particles as a result of using CTAB in the external phase (Oster et al., 2005). This may be due to differences in the formulation protocol. We did not observe any effect of changing the polymer type or polymer molecular weight on the morphology of nanoparticles.

3.3. Plasmid DNA loading on nanoparticles

Plasmid loading on the surface of cationic nanoparticles is primarily via ionic interaction, which depends on the zeta potential, though some non-ionic interaction may also exist. All the formulations displayed plasmid loading efficiency over 70% (Table 1). High plasmid loading efficiency of particles using CTAB as emulsifier in the external phase has been reported previously (Singh et al., 2000; Oster et al., 2005). In general, higher amount of pDNA loading was observed for particles with a higher surface zeta potential. Thus, nanoparticles prepared at a higher homogenization speed had higher plasmid loading as compared to those prepared at lower speed. Also, particles prepared with low molecular weight PLGA/PLA displayed higher loading efficiency as compared to those prepared with higher molecular weight PLGA/PLA (Table 1).

3.4. In vitro plasmid release

Figs. 2 and 3 show in vitro release of pDNA from cationic nanoparticles prepared from PLA and PLGA, respectively. Release of pDNA encapsulated into nanoparticles is governed primarily by the molecular weight of PLGA/PLA used. These polymers degrade by bulk hydrolysis forming progressively smaller polymer fragments, which eventually are solubilised. Lower molecular weight polymers, being more hydrophilic, degrade faster releasing the encapsulated molecule (Tinsley-Bown et al., 2000). In vitro plasmid release was, thus, expected to be faster for particles prepared with low molecular weight polymers due to greater hydrophilic nature coupled with a higher surface area as a result of smaller size. Contrary to this, low molecular weight polymers displayed a reduced burst release (amount released at day 1). This effect may be attributed to higher zeta potential of such particles leading to stronger ionic binding of pDNA resulting in lower burst release. Particles prepared at higher homogenization speed showed reduced burst release as compared to those prepared at lower speed. Higher zeta potential of particles prepared at higher speed results in stronger binding of negatively charged pDNA on the surface which causes lowering of burst release. Due to their better physicochemical properties and release behaviour, formulations prepared at higher speed (5000 rpm) were selected for further studies.

3.5. Structural integrity of plasmid DNA

Integrity of pDNA was studied both immediately after loading and in samples released at different time points, by agarose gel electrophoresis and was compared to untreated control DNA (Figs. 4 and 5). We found that pDNA extracted from nanoparticles immediately after loading showed good structural integrity. Majority of the extracted pDNA maintained its supercoiled conformation and was similar to the control DNA (Fig. 4). Samples released at different time points post-incubation were also evaluated for conformational stability of pDNA (Fig. 5). Plasmid was predominantly present in open circular form with some amount of supercoiled DNA. The proportion of supercoiled form was found to decrease further in samples taken at later time points. The open circular and supercoiled forms of pDNA are reported to be similar in their ability to transfect cells (Kimoto and Taketo, 1996). Thus, the nanoparticles were able to deliver pDNA in a functionally active form for a period of two weeks.

3.6. Cytotoxicity evaluation

Cationic agents have been reported to cause serious cellular toxicity due to electrostatic interaction with negatively charged cellular membrane (Fischer et al., 2003). In vitro toxicity of nanoparticles was evaluated by MTT assay in HEK293 cells using increasing doses of nanoparticles. Fig. 6 shows the results of MTT assay. The nanoparticle formulations were found to be non-toxic at majority of concentrations studied. We did not observe a significant difference ($p>0.05$) in the toxicity of different formulations at any of the concentrations used. There was a slight reduction in cell viabilities at higher concentrations. Average cell viabilities were between 80-120% of control at the concentrations studied.

3.7. Expression of beta-galactosidase

Ability of cationic nanoparticles to transfect cells in vitro was studied in HEK293 cells. Expression of beta-galactosidase was quantified by enzyme assay. We observed higher expression of beta-galactosidase in cells treated with low molecular weight polymer nanoparticles (Figs. 7A and 7B). This effect was observed for both PLGA and PLA based nanoparticles. Cationic particles have been reported to have a residual positive charge after adsorption of pDNA which leads to attachment of nanoparticles onto the surface of cells (Feng et al., 2006). The amount of residual charge present on the nanoparticles can determine their ability to adhere to the cell membrane and subsequently be taken up by the cells. Nanoparticles prepared with low molecular weight PLGA or PLA may have a higher amount of residual positive charge after loading of pDNA, due to their higher initial zeta potential, which may facilitate efficient cellular internalization of nanoparticles. Higher zeta potential coupled with smaller size may be responsible for higher transfection efficiency of cationic nanoparticles prepared with smaller molecular weight PLGA/PLA. We also studied the effect of storage of nanoparticles on transfection efficiency by comparing the efficiency of freshly made particles to those stored for two weeks at -20°C . We did not observe a significant ($p>0.05$) change in transfection efficiency after storage for two weeks at -20°C . This shows that cationic nanoparticles remain stable and preserve their transfection efficiency as well as the integrity of plasmid DNA for at least two weeks during storage at -20°C .

4. Conclusions

This study investigated the effect of changing formulation parameters on the properties of pDNA loaded cationic nanoparticles. Physicochemical properties of cationic nanoparticles such as size, zeta potential, plasmid loading efficiency and in vitro plasmid release were affected by polymer molecular weight and speed of homogenization. Loading on the surface of nanoparticles did not adversely affect the structural integrity of pDNA. Nanoparticle formulations displayed low toxicity in vitro. Lowering the molecular weight of PLGA/PLA led to a significant enhancement in the transfection efficiency of nanoparticles. In summary, it is feasible to deliver pDNA for prolonged duration by loading it on the surface of PLGA/PLA cationic nanoparticles and the physicochemical and biological properties of nanoparticles can be altered by changing the formulation variables.

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Fig.1.

Scanning electron micrographs of cationic nanoparticles prepared at 5000 rpm using polymers PLA0.20 (A), PLGA 0.44 (B), PLA 0.67 (C), and PLGA 0.63 (D).

Fig.2.
In Vitro release of plasmid DNA from nanoparticles prepared from different molecular weights of PLA at two different homogenization speeds (n=3).

Fig.3.

In Vitro release of plasmid DNA from nanoparticles prepared from different molecular weights of PLGA at two different homogenization speeds (n=3).

Fig.4. Agarose gel electrophoresis of pDNA extracted from cationic nanoparticles. Lane 1 corresponds to control pDNA. Lane 2-5 correspond to pDNA extracted from formulations 1, 3, 5, and 7, respectively. The lower band is supercoiled (S.C.) pDNA and the upper band is open circular (O.C.) pDNA.

Fig.5.

Agarose gel electrophoresis of pDNA released from formulation 1. Lanes 1-5 correspond to pDNA released at 0.5, 1, 3, 7, and 14 days, respectively. The lower band is supercoiled (S.C.) pDNA and the upper band is open circular (O.C.) pDNA.

Fig.6.
Cytotoxicity of cationic microparticle formulations analyzed by MTT assay, 24 h post treatment (n=8).

Fig.7.

Expression of beta-galactosidase by PLA (A) and PLGA (B) nanoparticles, fresh (Black columns) or after storage for two weeks (White columns). Nanoparticles were incubated at a dose equivalent to 500ng pDNA per well. Plasmid DNA in equivalent dose was used as control (n=4).

Table 1:

Effect of polymer molecular weight (inherent viscosity) and homogenization speed on physical properties of cationic nanoparticles

Formulation	Polymer	Mean particle size (nm)	Polydispersity index (PDI)	Zeta Potential (mV)	Loading efficiency*
1	PLA 0.20 ^a	737	0.39	58.50 ± 0.71	97.84%
2	PLA 0.20 ^b	887	0.37	48.42 ± 1.20	80.15%
3	PLA 0.67 ^a	769	0.34	31.86 ± 1.18	96.61%
4	PLA 0.67 ^b	1142	0.28	26.84 ± 0.74	80.45%
5	PLGA 0.44 ^a	740	0.34	53.40 ± 0.68	94.44%
6	PLGA 0.44 ^b	919	0.26	24.56 ± 1.74	72.15%
7	PLGA 0.63 ^a	795	0.43	27.62 ± 0.83	92.61%
8	PLGA 0.63 ^b	1000	0.27	24.86 ± 0.87	76.10%

^a Homogenization speed 5000 rpm

^b Homogenization speed 3000 rpm

* Loading efficiency calculated as a percentage of theoretical loading of 10µg pDNA/mg nanoparticles