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Formulation and In Vitro Transfection Efficiency of Poly (D, L-lactide-co-glycolide) Microspheres Containing Plasmid DNA for Gene Delivery

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ABSTRACT The stability, in vitro release, and in vitro cell transfection efficiency of plasmid DNA (pDNA) poly (D,L-lactide-co-glycolide) (PLGA) microsphere formulations were investigated. PLGA microspheres containing free and polylysine (PLL)-complexed pDNA were prepared by a water-oil-water solvent extraction/evaporation technique. Encapsulation enhanced the retention of the supercoiled structure of pDNA as determined by gel electrophoresis. PLL complexation of pDNA prior to encapsulation increased both the stability of the supercoiled form and the encapsulation efficiency. Free pDNA was completely degraded after exposure to DNase, while encapsulation protected the pDNA from enzymatic degradation. Rapid initial in vitro release of pDNA was obtained from microspheres containing free pDNA, while the release from microspheres containing PLL-complexed pDNA was sustained for more than 42 days. Bioactivity of encapsulated pDNA determined by in vitro cell transfection using Chinese hamster ovary cells (CHO) showed that the bioactivity of encapsulated pDNA was retained in both formulations but to a greater extent with PLL-complexed pDNA microspheres. These results demonstrated that PLGA microspheres could be used to formulate a controlled-release delivery system for pDNA that can protect the pDNA from DNase degradation without loss of functional activity.

Key Words: PLGA, Microspheres, Plasmid DNA, Controlled Release

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INTRODUCTION

Plasmid DNAs are being developed for gene therapy, vaccination, and other applications [1] . To date, DNA delivery technology has focused on free DNA delivery (as in DNA vaccination) and viral vector–mediated systems. Safety issues such as immunogenicity and mutagenic potential have delayed clinical applications of viral-mediated systems [2] . The generally poor efficiency of delivery and expression by non-viral systems are limitations in the development of gene therapy [3]. Thus, current research efforts are being focused on the design of new formulations of pDNA with lipids [4-9], polypeptides [10], and other complexes [11, 12]. However, these systems deliver DNA as a bolus, without long-term sustained release. Because interstitial administration of a single dose of free DNA can lead to a transient gene expression, it is reasonable to speculate that prolonged, continuous DNA delivery to tissues will enhance gene expression [1].

Biodegradable microspheres of poly (D,L-lactide-co-glycolide) (PLGA) have been widely investigated for the delivery of therapeutic agents ranging from conventional small molecules to large macromolecules. PLGA microspheres have shown promising results in the delivery of many therapeutic peptides [13-15]. Microspheres of PLGA are biodegradable and biocompatible, and they offer many advantages over conventional delivery systems. Encapsulation of therapeutic agents such as DNA in microspheres protects the agent from enzymatic degradation, enhances tissue specificity due to localized delivery, eliminates the need for multiple administration, and allows for controlled and sustained delivery. The

preparation and characterization of microspheres containing pDNA were reported previously [16]. Also reported has been the formation of a complex between pDNA and polylysine (PLL) to increase the stability of pDNA [17]. PLL is a positively charged polypeptide that interacts by charge interaction with pDNA, which has an overall negative charge due to the presence of multiple phosphate groups. The goal of this study was to formulate free and PLL-complexed pDNA in PLGA microspheres and evaluate the stability, release, and in vitro cell transfection efficiency of this delivery system.

MATERIALS AND METHODS

Materials

Valentis, Inc. (Burlingame, CA) provided pDNA (supercoiled, ~5 kilobase [kb])encoding luciferase, and 50:50 PLGA Resomer® RG503 (MW 30 KDa) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (MW 36-70 K) and PLL (MW 25 K) were supplied by Sigma Chemical (St. Louis, MO). PicoGreen® reagent was purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained commercially as analytical-grade reagents.

Preparation of microspheres

Microspheres were prepared by a water-oil-water (w/o/w) emulsion solvent evaporation/extraction method. Briefly, the pDNA:PLL complex (1:0.6, wt/wt) was prepared at room temperature by gently mixing 200 µ L of pDNA (2.25-6.75 mg/mL) in Tris-EDTA buffer (TE; 10 mmol/L Tris-HCl buffer containing 1 mmol/L EDTA, pH 7.4) with an equal volume of PLL (1.35-3.75 mg/mL) in TE buffer. The formation of the pDNA:PLL complex was confirmed by PicoGreen dye exclusion assay and agarose gel electrophoresis with ethidium bromide staining. Then 150 mg of PLGA was dissolved in 1.8 mL of methylene chloride (6% PLGA/methylene chloride, wt/wt) and 400 µ L of pDNA:PLL complex was added and mixed by vortexing for 2 minutes at room temperature. The primary water-oil dispersion was injected with a syringe into an aqueous 4% (wt/vol) polyvinyl alcohol (PVA) solution containing 10% sucrose (continuous phase) at 15°C while being mixed with a Silverson Laboratory Mixer-L4R (Silverson Machines, Inc., East Longmeadow, MA). The solvent was extracted by transferring the resulting w/o/w emulsion to 150 mL of 0.35% PVA solution containing 10% sucrose and stirring for 1 hour at 37°C. The solidified microspheres were recovered by filtration and dried under vacuum at room temperature.

Particle size distribution

Particles were sized by laser diffractometry using a Malvern 2600 laser sizer (Malvern Instruments PC6300, Malvern, England) .The average particle size was expressed as the volume mean diameter (v_{md}) in µ m.

Surface morphology

The surface morphology was examined by scanning electron microscopy (Hitachi Model S800, Tokyo, Japan) after palladium/gold coating of the microsphere sample on an aluminum stub.

Zeta potential measurements

The zeta potential of pDNA:PLL complexes formed with increasing amounts of PLL were determined using an electrophoretic light-scattering technique (Zetasizer 2000, Malvern Intruments, Ingleside, IL). Complexes were formed in water by adding increasing amounts of PLL to a constant amount of pDNA, in which PLL/pDNA (wt/wt) ratios were 0.0, 0.5, 1.0, 2.0, and 4.0. The reaction mixtures were kept at room temperature for 30 minutes before zeta-potential reading. Three measurements were performed on each sample and data shown are mean average values.

Molecular weight of polymer

The molecular weight of polymer during in vitro incubation of microspheres at 37ºC was measured by gel permeation chromatography (GPC). The GPC system consisted of 2 GPC columns connected in series (Ultrastyragel 10^4 Å and µ styragel 10^3 Å), solvent

(Tetrahydrofuran [THF], HPLC grade), delivery device (Shimadzu LC-6A, flow rate 1 mL/min), refractive index detector (Perkin Elmer, LC-25), and software to compute molecular weight distribution (Waters, Maxima 820, Medford, MA). Narrow band polystyrene standards were used for calibration. Sample solutions were prepared in THF at a concentration of 0.1% and filtered through a 0.45 μ m filter before injection into the GPC system.

Determination of pDNA loading of microspheres

The integrity of the pDNA:PLL complex was determined by dissolving 5 mg of microspheres in 500 μ L of chloroform. To extract the complex, 200 μ L of 10 mmol/L Tris-HCl buffer (pH 7.4) was added. Following centrifugation at 3600 rpm for 3 minutes, the aqueous layer containing the pDNA:PLL complex was transferred to a fresh tube. The amount and conformation of the extracted pDNA:PLL complex was analyzed to determine encapsulation efficiency and potential alterations in plasmid structure. The pDNA:PLL complex loading was determined by fluorescence spectrophotometry (H-2000 Hitachi, Tokyo, Japan) using PicoGreen reagent. pDNA stability and topology were assessed by 0.8% agarose gel electrophoresis (Pharmacia Fine Chemicals, Stockholm, Sweden) using 40 mmol/L Tris-acetate buffer containing 1 mol/L EDTA and ethidium bromide staining. The ratio of supercoiled to degraded open-circular pDNA was quantitated by densitometry using a digital imaging system (Kodak digital science, Electrophoresis Documentation and Analysis System 120, Rochester, NY).

In vitro release study

The release experiments were carried out in 33 mmol/L phosphate buffer (pH 7.4). Ten milligrams of microspheres were suspended in 10 mL of phosphate buffer in 15 mL conical centrifuge tubes and incubated at 37°C. At predetermined time intervals, 1 mL aliquots of the supernatant were removed after gently shaking by hand followed by 5 minutes of centrifugation at 2000 rpm. The release medium was replaced with fresh buffer after each sampling. The concentration of pDNA in the supernatant was determined by fluorescence spectrophotometry using the PicoGreen dye assay. Two standards (ie, free and complexed pDNA [1:0.6 wt/wt complexation]) were used to construct a standard curve to determine in vitro release of free and complex-containing microspheres, respectively. Two measurements were performed on each in vitro release sample, and data presented are the mean average values.

DNase I digestion study

Five milligrams of microspheres were dispersed in 100 μ L of 10 mmol/L Tris-HCl buffer (pH 8.0) and 80 μ L of 10 mmol/L $MgSO_4$ in water. Then 20 μ L of 5 μ g/mL DNase I in 0.9% NaCl was added and incubated for 30 minutes at 37°C. After digestion, microspheres were washed 3 times with 200 μ L of fresh Tris-EDTA bufferand the pDNA:PLL complex was extracted and quantitated by PicoGreen assay.

In vitro cell transfection

CHO (Chinese hamster ovary) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained at 37ºC in α -MEM (alpha minimum essential medium) media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells were plated at a density of 2 x 10^5 cells per well into 24 well plates and allowed to grow for 24 hours before transfection. DOTAP/DOPE (2-dioleoyl-sn-glycero-3-trimethylammonium-propane/dioleyl phosphatidylethanolamine) was complexed with a fixed amount of pDNA (3 μ g) extracted from microspheres (1:1 wt/wt lipid:pDNA) in serum-free media for 30 minutes at room temperature. DOTAP/DOPE is a transfection reagent used in cell transfection experiments. The treatments were added to the CHO cells; 4 hours after incubation the serum-free media was removed and replaced with serum-containing media. Twenty-four hours post-transfection, the cells were washed with phosphate buffered saline (PBS) and lysed in 100 μL of luciferase lysis buffer (0.1 mol/L potassium phosphate buffer, pH 7.8, 2

mmol/L EDTA, 1% Triton X-100, 1 mmol/L dithiothreitol) at room temperature on a shaker for 15 minutes. The luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) by adding 100 µ L luciferase assay buffer (30 mmol/L tricine, 3 mmol/L ATP, 15 mmol/L $MgSO_4$, 10 mmol/L dithiothreitol, pH 7.8) to 20 µ L cell lysate and injecting 100 µ L of 1 mmol/L D-luciferin, pH 6.3. The emitted light was measured for 10 seconds and expressed in terms of relative light units (RLU) per well.

RESULTS AND DISCUSSION

Characteristics of pDNA microsphere formulations

Table 1 compares the properties of 2 microsphere formulations that contain pDNA and pDNA complexed with PLL. The pDNA content of the microspheres doubled from 0.17% to 0.34% when pDNA was complexed with PLL prior to encapsulation. Similarly, the supercoiled pDNA content of the pDNA:PLL microspheres (48.2%) was higher than microspheres containing only pDNA (37.3%). The supercoiled and open-circular contents were obtained by digital scanning of gel electrophoresis and comparing the band intensity of the supercoiled band (lower band) and the open-circular band (upper band) of lanes 4 and 5 in **Figure 3** as described in the Materials and Methods section. The particle-size distribution of the microspheres as measured by laser light particle–sizing technique ranged between 2.4 and 2.9 µ m with a mean average diameter of 2.6 µ m. The particle-size distribution obtained is ideal for targeting phagocytic cells such as macrophages. The percent yield of the 2 formulations (ie, formulation 1, microspheres containing only pDNA, and formulation 2, microspheres containing the pDNA:PLL complex) was also similar at a value of between 70% and 75%. The major factor that contributed to a decrease of the percent yield value from the ideal 100% was the recovery step. The microspheres were recovered by filtering the continuous phase at the end of the microsphere formation process through a 0.45 µm filter under nitrogen pressure and removing the particles

Table 1. Physical properties of pDNA containing microspheres

Description	Formulation	
	F1	F2
pDNA:PLL (w/w)	1:0	1: 0.6
pDNA target load (%)	1.0	1.0
Drug content (%)	0.17	0.34
Yield (%)	75.5	72.5
Incorporation efficiency (%)	17.0	33.7
Supercoiled pDNA content (%)	37.3	48.2
Open-circular pDNA content(%)	62.7	52.1
Particle size (µ m)	2.6	2.4

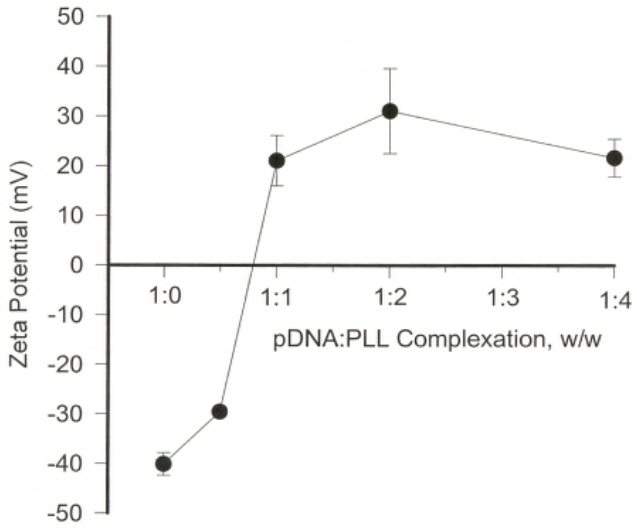

Figure 1. The effect of pDNA:PLL complexation on Zeta potential of complexes

retained on the filter paper. This filtration process is very efficient at retaining close to 100% of the microspheres. However, some of the microspheres get stuck on the filter paper. Removal of the microspheres retained on the filter paper is not 100% efficient because some of the particles get attached to the filter paper. This is particularly true if the filter paper is

allowed to dry excessively before removal of the particles. The pDNA incorporation efficiency doubled from 17.0% to 33.7% when pDNA was complexed with PLL. This apparent increase in both drug content and incorporation efficiency is due to the fact that complexation of pDNA with PLL neutralizes the negative charge of pDNA . **Figure 1** shows the results of the zeta-potential measurement of pDNA:PLL complexes.

As expected, free pDNA exhibits a negative zeta potential (-40 mV). In the presence of PLL, the zeta potential shifted to positive values and reached a maximum (+20 to +30 mV) at high PLL concentrations. From **Figure 1**, the zeta potential of 1:0.6 wt/wt complex is extrapolated to be slightly negative (-10 mV). However, compared with the free pDNA charge (-40 mV), the complex exhibits less negative charge and is closer to the neutralization zone. It is proposed that this charge neutralization of pDNA results in the complex becoming less hydrophilic, resulting in less of the pDNA escaping into the aqueous continuous phase during formulation of microspheres. By the same token, complexes at high PLL content show positive zeta-potential measurements and would be expected to have hydrophilic properties. Hence, in order to increase encapsulation efficiency, a pDNA:PLL complexation value that would result in zeta potential at or close to the neutralization zone is desirable. A maximum in zeta potential was reached at about 1:1 pDNA:PLL ratio, the ratio at which saturation binding was shown to occur [17].

Scanning electron microscopy (SEM) (**Figure 2**) shows that the microspheres have a smooth spherical surface. However, beneath the external coating surface, the internal structure of the microspheres is porous. The particle size of the microspheres as seen from the SEM is less than 5 μ m. Mean particle size of the microspheres as measured by the laser particle sizer was 2.4-2.6 μ m. This particle size distribution is a very crucial parameter for pDNA microspheres. pDNA-containing microspheres are expected to be taken up by phagocytic cells. It has been extensively reported

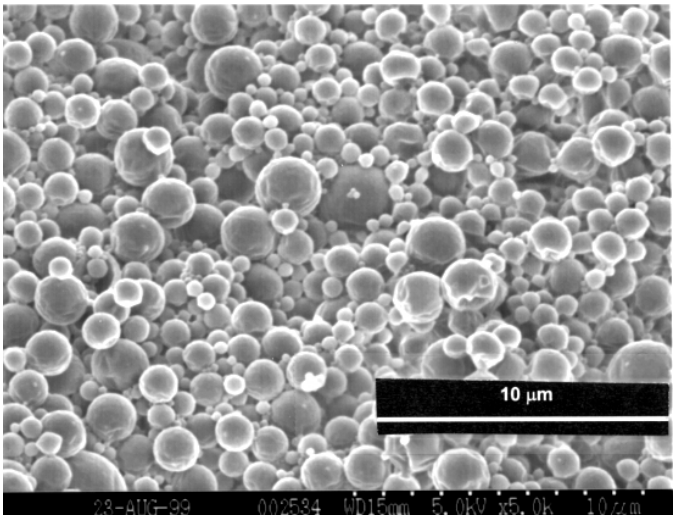

Figure 2. Scanning Electron Micrograph of PLL complexed pDNA containing PLGA microspheres.

[18, 19] that in order for phagocytic cells to take up particles, the particles have to be smaller than 10 μ m. The microsphere preparation process was optimized to give the desired particle size distribution without compromising the structural and functional integrity of the pDNA. Homogenization speed and polymer viscosity were found to be crucial parameters that determined the particle size of microspheres containing pDNA. At PLGA polymer concentration of 6% wt/wt in methylene chloride, high initial homogenization at 7000 rpm for 1 second gave desired particle size distribution. The residual methylene chloride was extracted at a slower homogenization speed of 500 rpm. It is important to limit the exposure of pDNA to high-speed homogenization because pDNA is sensitive to conversion from supercoiled to open-circular form upon shear stress. The microspheres are formed immediately upon contact of the dispersed phase with the continuous phase with a particular particle morphology and size distribution. Therefore, optimization of initial parameters is crucial. The supercoiled structure of pDNA has been reported in the literature [20] to be the more bioactive form. Agarose gel electrophoresis analysis of pDNA extracted from the microspheres (**Figure 3**) shows that the supercoiled structure was retained in the microspheres.

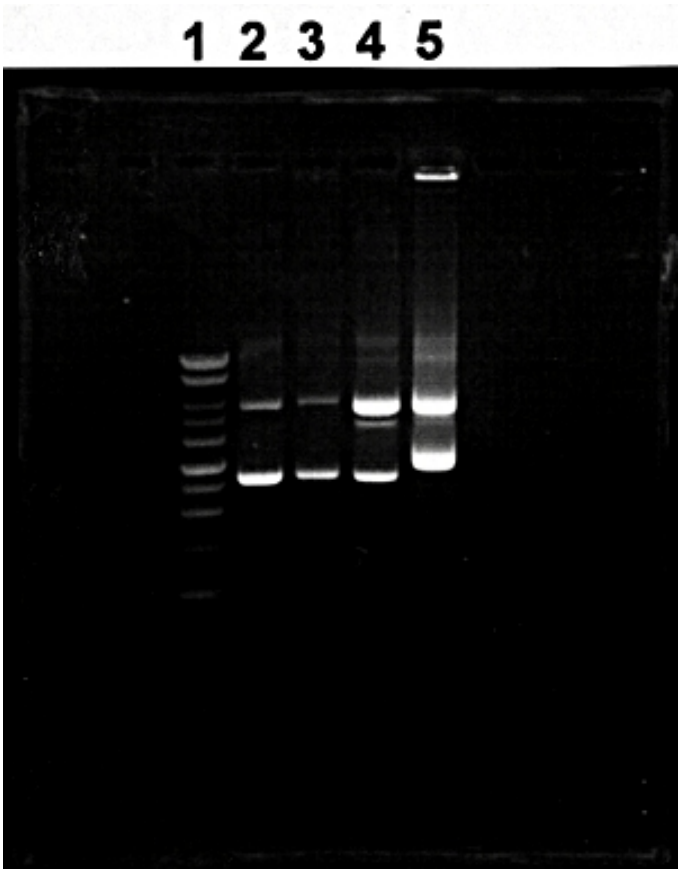

Figure 3. Agarose gel electrophoresis of unencapsulated and encapsulated pDNA. Lane 1: 1Kb molecular weight ladder; lane2: unencapsulated free pDNA standard; lane 3: unencapsulated pDNA: PLL complex standard; lane 4: encapsulated pDNA; lane 5: encapsulated pDNA: PLL complex.

The agarose gel electrophoresis shows that free plasmid DNA (lane 2) exists in 2 forms: the supercoiled (lower band) and open-circular (upper band). The 2 forms of pDNA separate into 2 distinct bands on exposure to electric voltage. Supercoiled pDNA, being more compact than the open-circular form, travels faster than the open-circular form through the agarose polymer network, resulting in two distinct bands. Lane 2 (free pDNA) and lane 3 (pDNA:PLL complex) in Figure 3 show that pDNA is primarily in the supercoiled form. In comparison, lane 4 (pDNA extracted from microspheres) shows that the pDNA still existed in the supercoiled and open-circular forms but had higher open-circular form content compared with free pDNA (lane 2). It is presumed that some of the supercoiled form converted to the open-circular form during formulation of the microspheres. Interestingly, complexation of pDNA with PLL led to an increase in the intensity of the supercoiled band (lane 5), suggesting that complexation inhibits the conversion of the supercoiled form to the open-circular form. A minor band at the spot well was also observed in lane 5, which is presumed to be a high molecular weight pDNA:PLL complex aggregate that did not travel down the gel. Interestingly, this phenomenon is only observed from complex extracted from the microspheres (lane 5) and is absent in the reference standard complex (lane 3). Further work needs to be done to determine whether this aggregate is formed during the extraction procedure or whether it exists as such in the microspheres. The data indicate, however, that although optimization of processing parameters during the formation of microspheres containing pDNA is important to avoid conversion of the supercoiled form to the open-circular form, it may not be sufficient by itself. Complexation of pDNA with PLL prior to the encapsulation process offers an added degree of protection of the supercoiled form of pDNA.

In vitro release

Figure 4 shows the in vitro release profile of microspheres containing pDNA and pDNA:PLL. The release of pDNA from microspheres containing free uncomplexed pDNA was characterized by a release of approximately 60% in 3 days. Slower sustained release was observed over the next 12 days and 80% of the drug was released after 15 days. On the other hand, the release of pDNA from microspheres containing PLL-complexed pDNA showed little initial burst release. Approximately 25% released in a linear fashion over 21 days. The release accelerated thereafter and approximately 70% was released after 42 days. Convective diffusion of pDNA through the porous structure of the microspheres appears to be the mechanism of release of pDNA from microspheres containing free uncomplexed pDNA. Most of the pDNA is released well before significant loss in mass and molecular weight of the polymer occurred (Figure 5).

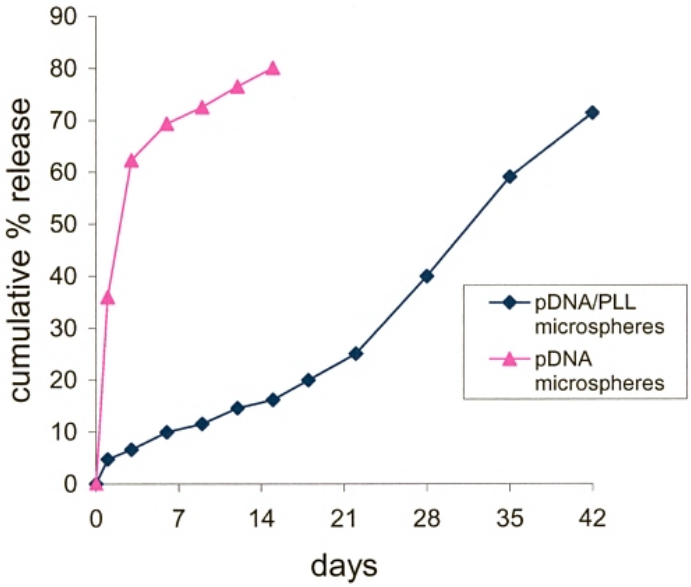

Figure 4. In vitro release of pDNA from microsphere formulations in phosphate buffer, pH 7.4, 37°C.

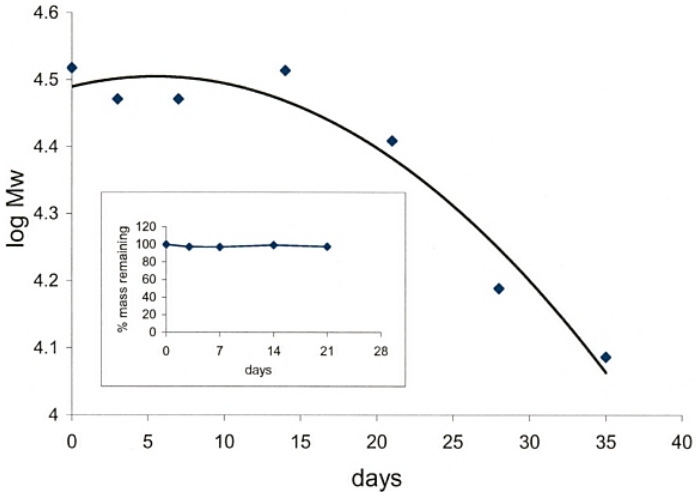

Figure 5. Change in the molecular weight and mass of PLGA polymer during in vitro incubation at 37°C.

Significant loss in both mass and molecular weight of the polymer matrix began after only about 3 weeks. The more sustained-release profile observed for microspheres containing PLL-complexed pDNA is presumed to be either a result of the lower solubility of complexed pDNA in the release medium or the increased association of the more hydrophobic complex with the hydrophobic polymer matrix.

Protection from enzymatic degradation

Free pDNA is rapidly degraded on exposure to DNase. The encapsulation of pDNA in PLGA microspheres protects pDNA from this enzymatic degradation. **Figure 6** shows the structure of free and PLGA microsphere–encapsulated pDNA after exposure to DNase enzyme. Gel electrophoresis of DNase-treated free pDNA (lane 4) shows complete degradation of the pDNA. On the other hand, PLGA encapsulation of pDNA in its free form or as a complex with PLL (lanes 5, 6) was able to retain the structural integrity of pDNA after exposure to DNase.

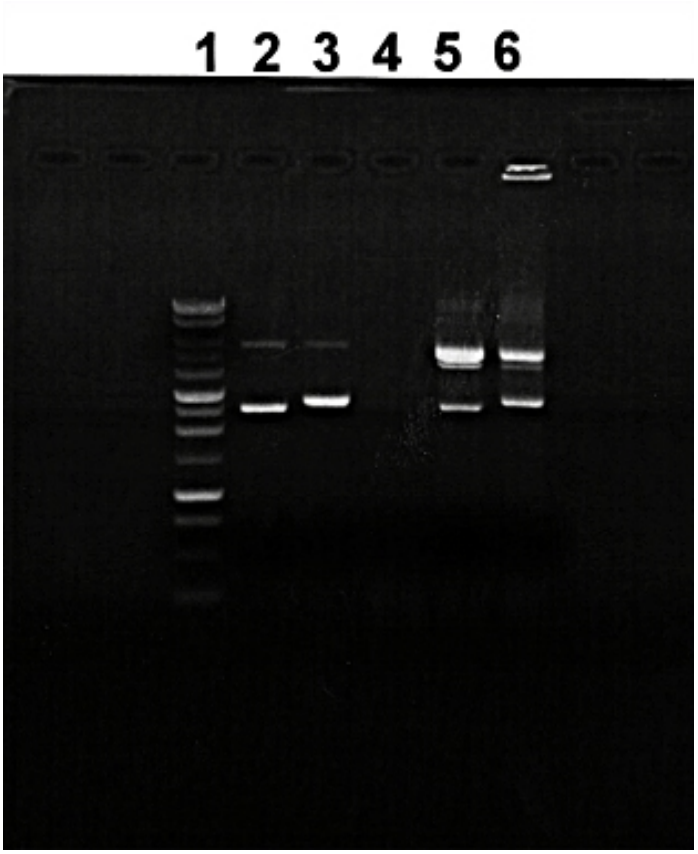

Figure 6. The effect of DNase on unencapsulated and encapsulated pDNA. Lane 1: 1Kb molecular weight ladder; lane2: unencapsulated free pDNA standard; lane 3: unencapsulated pDNA: PLL complex standard; lane 4: DNase treated free unencapsulated pDNA; lane 5: DNase treated encapsulated pDNA; lane 6: DNase treated encapsulated pDNA:PLL complex.

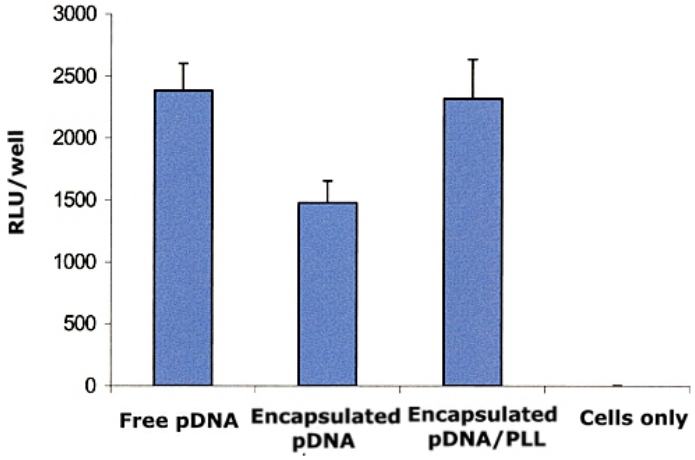

Figure 7. Luciferase activity of Cho cell lysates 24 hr. after in vitro transfection with pDNA extracted from PLGA microspheres and control pDNA. Untransfected CHO cell lysates were used as negative control. Luciferase activity is expressed in terms of relative light unit (RLU) per well. Error bars indicate S.D. (n=4).

In vitro cell transfection efficiency

The functional integrity of encapsulated pDNA was studied by an in vitro transfection assay using CHO cells. pDNA extracted from the microspheres was able to transfect this cell line as measured by the luciferase activity of the cell lysate (Figure 7). The transfection activity of encapsulated free pDNA was lower than that of the control free pDNA. However, complexation of pDNA to PLL prior to encapsulation resulted in a transfection activity similar to the control pDNA. This result suggests that the complexation of free pDNA with PLL was able to maintain the functional activity of pDNA, which seems to be affected during microsphere preparation.

CONCLUSION

Microencapsulation of pDNA using PLGA microspheres provides a promising method of formulating a stable delivery system for pDNA. The results of this study demonstrate that pDNA can be encapsulated in PLGA microspheres without degrading the bioactive supercoiled structure of pDNA. Complexation of pDNA with polylysine prior to encapsulation increases the stability of the pDNA during preparation of microspheres and increases the encapsulation efficiency. The in vitro transfection efficiency experiment indicated that PLGA-encapsulated pDNA is bioactive and able to transfect cells. These results warrant further studies on the in vivo evaluation of gene expression of microsphere formulations.

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