Preparation and Characterization of Poly (D,L-Lactide-Co-Glycolide) Microspheres for Controlled Release of Poly(L-Lysine) Complexed Plasmid DNA

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Received November 5, 1998; accepted January 12, 1999

Purpose. To produce and characterize controlled release formulations of plasmid DNA (pDNA) loaded in poly (D,L-lactide-co-glycolide) (PLGA) microspheres both in free form and as a complex with poly (L-lysine).

Methods. Poly (L-lysine) (PLL) was used to form pDNA/PLL complexes with complexation ratio of 1:0.125 and 1:0.333 w/w to enhance the stability of pDNA during microsphere preparation and protect pDNA from nuclease attack. pDNA structure, particle size, zeta potential, drug loading, in vitro release properties, and protection from DNase I were studied.

Results. The microspheres were found to be spherical with average particle size of 3.1–3.5 μm. Drug loading of 0.6% was targeted. Incorporation efficiencies of 35.1% and 29.4–30.6% were obtained for pDNA and pDNA/PLL loaded microspheres respectively. Overall, pDNA release kinetics following the initial burst did not correlate with blank microsphere polymer degradation profile suggesting that pDNA release is convective diffusion controlled. The percentage of supercoiled pDNA in the pDNA and pDNA/PLL loaded microspheres was 16.6% and 76.7–85.6% respectively. Unencapsulated pDNA and pDNA/PLL degraded completely within 30 minutes upon the addition of DNase I. Encapsulation of DNA/PLL in PLGA microspheres protected pDNA from enzymatic degradation.

Conclusions. The results show that using a novel process, pDNA can be stabilized and encapsulated in PLGA microspheres to protect pDNA from enzymatic degradation.

KEYWORDS: controlled release; microspheres; DNA; DNA/poly(L-lysine) complex; poly(lactide-co-glycolide).

INTRODUCTION

For gene therapy to become a reality, safe and effective methods must be found to deliver DNA efficiently to target cells (1). A number of techniques have been developed for the introduction of genes into cells. Although delivery systems of viral origin, such as retrovirus (2,3) and adenovirus (4), efficiently introduce genes, they suffer from immunogenicity, toxicity, and lack of tissue specificity. The immunogenicity of viral vectors restricts the repeated use of the delivery systems. Non-viral delivery systems, such as cationic lipids, liposomes and polymeric microspheres have been increasingly proposed as alternatives to viral vectors because of potential advantages such as tissue-specific targeting, relative ease of large-scale production, and relative safety (5–7).

Free pDNA is rapidly fragmented within 30 min during in vitro mouse serum incubation due to the action of endonucleases (8). Adami et al. showed that the stabilization afforded by condensation with a peptide protects DNA during formulation and preserves its structure in serum (9). pDNA is also rapidly eliminated from the plasma when injected intravenously into mice without any delivery system (10).

Biodegradable polymers have shown promising results in the delivery of many bioactive peptides (11). The development of biodegradable microspheres for pDNA delivery may offer several advantages over other formulations. First, encapsulation of pDNA in microspheres could protect DNA from rapid in vivo degradation. Secondly, localized delivery of pDNA may increase the amount of pDNA retained within tissues (12).

We are interested in describing a delivery system where PLL complexed pDNA is incorporated in PLGA microspheres. The aim of this study is to determine whether PLL complexed pDNA can be incorporated and continuously released from PLGA microspheres without degradation. The characteristics of the microspheres were examined under various conditions. Characterization included analyzing released pDNA for conformational/structural integrity and release kinetics.

MATERIALS AND METHODS

Materials

pDNA (supercoiled, ~5Kb) was provided by Pangaea Pharmaceuticals Inc. 50:50 poly(D,L-lactide-co-glycolide) (m.w. 32,510, Resomer® RG503, PLGA) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (m.w. 30,000–70,000, PVA) and poly(L-lysine) (m.w. 25,000, PLL) were supplied by Sigma Chemical (St Louis, MO, USA). Pico Green® reagent was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were obtained commercially as analytical grade reagents.

Preparation of Microspheres

Microspheres of PLGA were prepared by modification of a previously described procedure (13). pDNA/PLL complex was prepared by the rapid mixing of 900 μg of pDNA in Tris-EDTA buffer with varying amounts of PLL. In this study two pDNA/PLL complexes (pDNA/PLL ratio, 1:0.125 and 1:0.333, w/w) were used for the preparation of microspheres. Complex formation was allowed to proceed spontaneously at room temperature for 30 min. Experiments were performed to investigate whether PLL forms a complex with pDNA. Physicochemical characterization of the pDNA/PLL complex was examined by PicoGreen® dye exclusion assay and agarose gel electrophoresis with ethidium bromide staining as described below. The complex was prepared immediately prior to the experiments. The microspheres were prepared by dispersing an aqueous solution of pDNA or pDNA/PLL complex into a 6% (w/w) solution of PLGA dissolved in methylene chloride followed by 2min. vortex mixing. The primary w/o dispersion was injected with a syringe into an aqueous 4% PVA solution containing 10% sucrose (continuous phase) at 15°C while being mixed with a

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Silverson laboratory mixer-L4R (Silverson Machines Inc., USA.). The solvent was extracted by transferring the resulting w/o/w emulsion into 150 ml of 0.35% PVA solution containing 10% sucrose and stirring for 1 hr at 37°C. The solidified microspheres were recovered by filtration and dried under vacuum at room temperature.

**Particle Characterization**

**Particle Size Distribution**

Particles were sized by laser diffractometry using a Malvern 2600 laser sizer (Malvern Instruments PC6300, England). The average particle size was expressed as the volume mean diameter, \( \bar{y}_{vd} \), in \( \mu \text{m} \).

**Surface Morphology**

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

**Zeta Potential Measurement**

Zeta potential of microspheres was measured in 10 mM Tris-HCl buffer (pH 7.4) using a Zeta Meter (Zeta Meter, Inc.) equipped with a Nikon SMZ-2 stereoscopic microscope. Velocity measurements were performed on 10 individual particles at a voltage of 150 V and the zeta potential was calculated using the average velocity of 5 runs.

**Determination pDNA Loading of Microspheres**

PLGA microspheres were dissolved in chloroform. pDNA or pDNA/PLL complex was extracted from the polymer solution by addition of 10 mM Tris-EDTA buffer (pH 7.4). The amount and conformation of the extracted pDNA, or pDNA/PLL complex was analyzed to determine encapsulation efficiency and potential alterations in plasmid structure. The quantification and conformational measurement of the pDNA and the pDNA/PLL complex will be reported elsewhere (unpublished data). Briefly, pDNA loading was determined by Fluorescence spectrophotometry (H-2000 Hitachi, Japan) using Picogreen® reagent. pDNA stability and topology were assessed by 0.8% agarose gel electrophoresis (Pharmacia Fine Chemicals, Sweden) using 40 mM Tris-acetate buffer containing 1 mM EDTA (TAE) and ethidium bromide staining. The ratio of supercoiled to degraded linear pDNA was quantitated densitometrically using a Kodak Scanner (Kodak digital science, Electrophoresis Documentation and Analysis System 120).

**In Vitro Release Study**

The release experiments were carried out in 33 mM phosphate buffer (pH 7.4). 10 milligrams of microspheres were added to 10 mL of phosphate buffer in 15 mL conical centrifuge tubes incubated at 37°C. At predetermined time intervals, 1 mL aliquots of the supernatant were removed. The dissolution 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. Released pDNA or pDNA/PLL complex was analyzed by agarose gel electrophoresis and compared to unencapsulated stock pDNA or pDNA/PLL complex to determine the conformation of released pDNA.

**Mass Loss Study**

50 milligrams of blank PLGA microspheres were added to 50 mL of 33 mM phosphate buffer (pH 7.4) in 50 mL conical centrifuge tubes incubated at 37°C. At different time intervals, the tubes were removed and the microspheres were collected by filtration (0.22 \( \mu \text{m} \), Millipore). The microspheres were vacuum dried and weighed.

**DNase I Digestion Study**

pDNA, pDNA/PLL complexes and pDNA microspheres were incubated with 5 \( \mu \text{g} \) of DNase 1 in 10 mM Tris-HCl buffer containing 10 mM MgSO\(_4\) (pH 8.0) for 30 min at 37°C. Following digestion, samples were analyzed by 0.8% agarose gel electrophoresis for DNA fragments. Kodak scanning was used to quantitate the ratio of supercoiled to linear pDNA.

**RESULTS AND DISCUSSION**

**Preparation of pDNA/PLL Complex**

Picogreen® dye exclusion proved to be a useful indicator of complexation between PLL and pDNA. As PLL was added to pDNA, the fluorescence emitted decreased until a minimum level was reached close to baseline fluorescence. PLL was shown to form a complex with pDNA where up to 95% of the original fluorescence intensity was quenched. Gel retardation assay showed that the movement of pDNA was retarded as the amount of PLL increased indicating that PLL forms a complex with pDNA (data not shown). The pDNA/PLL complexes show much weaker bands in intensity, probably due to the exclusion of ethidium bromide following formation of complexes. When increasing amounts of PLL were added to the pDNA, the pDNA band completely disappeared suggesting that the complexes formed were too large to migrate into the gel matrix or that pDNA was completely covered by PLL thereby preventing ethidium bromide staining.

**Effect of Processing on Microsphere Size and DNA Entrapment**

The process of microsphere preparation by the solvent evaporation method, using a double emulsion, involves several steps that can damage pDNA. Exposure to high interfacial tension at the methylene chloride/water interphase, ultrasonic radiation and vortex mixing during preparation of the primary w/o dispersion affect the stability of pDNA. Preliminary studies to examine the effect of each step in the preparation process on the total amount and the stability of pDNA showed that sonication using a probe sonicator (Heat Systems, Ultrasonics, Inc.) for 60 s, to form the primary w/o dispersion, has the most detrimental effect on pDNA stability. Following sonication, pDNA was completely degraded in the primary w/o dispersion (data not shown). Therefore, sonication was avoided in the preparation of the dispersed phase and vortex mixing was adopted. The addition of 10% sucrose to the PVA solution