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Gene delivery nanoparticles fabricated by supercritical fluid extraction of emulsions

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ABSTRACT

Non-viral polymeric gene delivery systems offer increased protection from nuclease degradation, enhanced plasmid DNA (pDNA) uptake, and controlled dosing to sustain the duration of pDNA action. Such gene delivery systems can be formulated from biocompatible and biodegradable polymers such as poly(D,L-lactic-co-glycolic) acid (PLGA). Experimental loading of hydrophilic macromolecules such as pDNA is low in polymeric particles. The study purpose was to develop a supercritical fluid extraction of emulsions (SFEE) process based on CO_2 for preparing pEGFP-PLGA nanoparticles with high plasmid loading and loading efficiency. Another objective was to determine the efficacy of pFlt23k, an anti-angiogenic pDNA capable of inhibiting vascular endothelial growth factor (VEGF) secretion, following nanoparticle formation using the SFEE process. Results indicated that the SFEE process allows high actual loading of pDNA (19.7%, w/w), high loading efficiency (>98%), and low residual solvents (<50 ppm), due to rapid particle formation from efficient solvent removal provided by the SFEE process. pFlt23K-PLGA nanoparticles were capable of *in vitro* transfection, significantly reducing secreted VEGF from human lung alveolar epithelial cells (A549) under normoxic and hypoxic conditions. pFlt23K-PLGA nanoparticles did not exhibit cytotoxicity and are of potential value in treating neovascular disorders wherein VEGF levels are elevated.

1. Introduction

Even though the therapeutic potential of plasmid DNA (pDNA) is well recognized, the use of plasmid DNA-based pharmaceuticals is limited by the development of safe and efficient gene delivery systems (Friedmann, 1997). With growing interest in nano-sized systems, there have been concentrated efforts to create efficient non-viral gene delivery vectors. Commonly used non-viral gene delivery vectors are matrix-type polymeric systems composed of biodegradable and biocompatible polymers such poly(D,L-lactic-co-glycolic) acid (PLGA), poly(lactic) acid (PLA), and chitosan. PLGA-nanoparticle gene delivery systems have the ability to enter cells via an energy-dependent, endocytotic process followed by rapid escape from secondary endosomes into the cytoplasm (Panyam et al., 2002).

One limitation of the existing approaches for non-viral gene delivery nanoparticles prepared using polymers such as PLGA is the low experimental loading and low loading efficiency for hydrophilic macromolecules such as plasmids (Park et al., 2009). An additional issue with some particulate delivery systems is that residual organic solvents can remain above regulatory levels using conventional preparation methods (Thoma and Schlutermann, 1992). Investigators have reported the use of SCF technology to produce particulate delivery systems with low quantities of residual solvents (Koushik and Kompella, 2004; Bleich and Muller, 1996; Ruchatz et al., 1997). High levels of residual solvents may cause toxicity or impair the activity of labile macromolecules. Koushik and Kompella (2004) demonstrated that supercritical fluid (SCF) processes based on CO_2, while reducing the residual solvents to levels less than 25 ppm, preserved the active form of a peptide drug. Therefore, using pEGFP plasmid as a model, one objective of this study was to develop a supercritical CO_2 based process to prepare pDNA loaded PLGA nanoparticles with high loading efficiency, high experimental loading of plasmid, and low residual solvents.

SCF technology has demonstrated usefulness in the formation of stable pDNA pharmaceutical powders suitable for inhalation as well as needle-free injections (Tservistas et al., 2001). The technique of solution enhanced dispersion by supercritical fluids (SEDS) was used to formulate the 6.9 kb plasmid pSVβ with mannitol as a stabilizing excipient using a three-channeled coaxial nozzle. The processed plasmids allowed 80% recovery of the native supercoiled DNA and resulted in cellular transfection. Using a SEDS

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Table 1
SCF processes for plasmid DNA gene delivery systems.

Plasmid/polymer	Process/solvent	Flow rate (a) CO_2 (b) pDNA Soln (mL/min)	Temperature/pressure (°C/MPa)	Mean size (nm)	Product	Reference
pSVβ	SEDS/isoproanol	(a) 10 (b) 0.03	50/20	–	Dry plasmid powder with mannitol	Tservistas et al. (2001)
pCMV-Luc/chitosan	SEDS/ethanol	(a) 12.3 (b) 0.035	35/15	1200–1300	Rectangular shaped particles	Okamoto et al. (2003)

process, chitosan-pDNA complexes (mean diameter 12.2 μm) were formed for use in pulmonary gene delivery (Okamoto et al., 2003). The pDNA–polymer complex was effective for *in vivo* transfection studies, expressing the luciferase protein. Table 1 summarizes the results of previously reported work on SCF processing of pDNA particulate systems. However, these earlier studies did not achieve high experimental plasmid loading in the particles. Further, the particles were largely in the micron range. In this study, we aimed at preparing pEGFP-PLGA nanoparticles with high (~20%, w/w) plasmid loading. For this purpose, we developed a supercritical fluid extraction of emulsions (SFEE) method for gene delivery nanoparticle fabrication.

Chattopadhyay et al. (2006a,b; Shekunov et al., 2006), first successfully fabricated composite micro- and nanoparticles utilizing supercritical fluid extraction of emulsions (SFEE) for sustained-release drug formulations utilizing both batch and continuous processing. Model hydrophobic drugs such as indomethacin and ketoprofen were encapsulated in biodegradable PLGA and pH-independent swelling Eudragit RS polymers, forming composite particles ranging in size between 0.1 and 2.0 μm. The drug loading efficiency for composite particles was approximately 98% of the theoretical loading as determined by HPLC. The present study assessed the applicability of SFEE processes in preparing nanoparticles of hydrophilic plasmids as opposed to low molecular weight hydrophobic drugs. The advantage of SFEE processing compared to most conventional methods such as evaporation, solvent extraction, and dilution is the higher solvent extraction rate and efficiency. Higher extraction rates enable faster supersaturation, which forms a greater number of nuclei and more uniform particles (Baldyga et al., 2004). Additionally, SFEE processing is expected to increase plasmid DNA encapsulation efficiency as the process is a single step spray-type operation that does not involve pDNA loss due to extensive partitioning between the immiscible aqueous and lipophilic phases as is the case with the conventional solvent evaporation method.

Another objective of this study was to assess the efficacy of a plasmid-PLGA-nanoparticle formulation prepared using SFEE process. Multiple factors are responsible for lung tumor angiogenesis, but vascular endothelial growth factor (VEGF) has been identified as one of the most potent in this process (Dvorak et al., 1999). Additionally, VEGF expression in hypoxic corneal epithelial cells is elevated when compared to normal expression (Singh et al., 2005). Anti-VEGF intraceptor plasmid (pFlt23K) is a construct capable of expressing a protein, wherein the high affinity VEGF receptor 1 (Flt) domains 2 and 3 are coupled with an endoplasmic reticulum retention signal sequence (KDEL). This protein is expected to bind and retain VEGF within the endoplasmic reticulum of the target cell. Such protein retention ultimately leads to protein degradation (Pelham, 1990). Thus, gene delivery vectors of pFlt23k are expected to reduce cellular VEGF secretion and the consequent vascular hyper-permeability and/or angiogenesis. Therefore, in this study, pFlt23K-PLGA nanoparticles prepared using SFEE were assessed for their efficacy in cancerous cells (A549) of the human lung alveolar epithelium.

2. Materials and methods

2.1. Materials

PLGA 85:15 (Birmingham Polymers Inc., MW 90 kDa) was obtained and used as received. The coaxial SFEE apparatus consisted of an extraction vessel (Whiley, Solon, OH), a syringe pump (Suprex-Isco, Lincoln, NE), high pressure tubing and fittings (High Pressure Equipment Company, Erie, PA), a back pressure regulator (Tescom, Elk River, MN), a CO_2 source (99%, Lindweld, Omaha, NE), and a temperature controlled water bath (Fisher Scientific, Hampton, NH). Chemicals including ethyl acetate, sodium chloride, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma–Aldrich, St. Louis, MO of the highest grade commercially available. pEGFP plasmid was obtained from Promega, Madison, WI. pFlt23K was a gift from Dr. Bala Ambati at University of Utah, Salt Lake City, UT. Pico Green Assay Kit was obtained from Molecular Probes, Carlsbad, CA.

2.2. Amplification and purification of plasmid DNA

The pFlt23K or pEGFP plasmid was transformed into competent *Escherichia coli* (DH5-α). Cells were then amplified in Luria–Bertani (LB) media at 37 °C, 250 rpm overnight and centrifuged to obtain a bacterial pellet. The plasmid was purified using a QIAGEN Giga Plasmid Purification Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Purified DNA was dissolved in sterile Tris–EDTA buffer (pH 8.0), and its purity and concentration were determined by UV absorbance at 260 and 280 nm.

2.3. Cell culture

A549 (ATCC, CCL 185) cells were cultured in F12K medium containing 10% fetal bovine serum, 50 U/mL penicillin G, and 50 μg/mL streptomycin sulfate. Cells were maintained at 37 °C under a 5% CO_2 and 95% O_2 atmosphere in T-75 flasks. Transfection studies were performed with cells cultured in 96-well plates at 60% confluency, while cytotoxicity assays were performed at 100% confluency.

2.4. Emulsion preparation

The lipophilic phase was prepared by dissolving 55 mg or 18 mg of PLGA 85:15 into 2 mL of ethyl acetate (3%, w/w_{EA}, 1%, w/w_{EA}, respectively). The inner aqueous phase composed of 260 μL (1.1 mg pFlt23K) or 544 μL (3.6 mg pEGFP) TE buffer was combined with the lipophilic phase and sonicated for 1 min (15 W) to form a primary w/o emulsion. An outer aqueous phase of 18 mL 0.5% (w/v) poly(vinyl alcohol) (PVA) was filtered through a 0.22 μm syringe filter and saturated with ethyl acetate. The primary emulsion was further emulsified with the outer aqueous phase for 3 min (42 W) forming a w/o/w emulsion. Emulsification was performed on an ice bath to prevent excessive heating that could denature the pDNA.