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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.

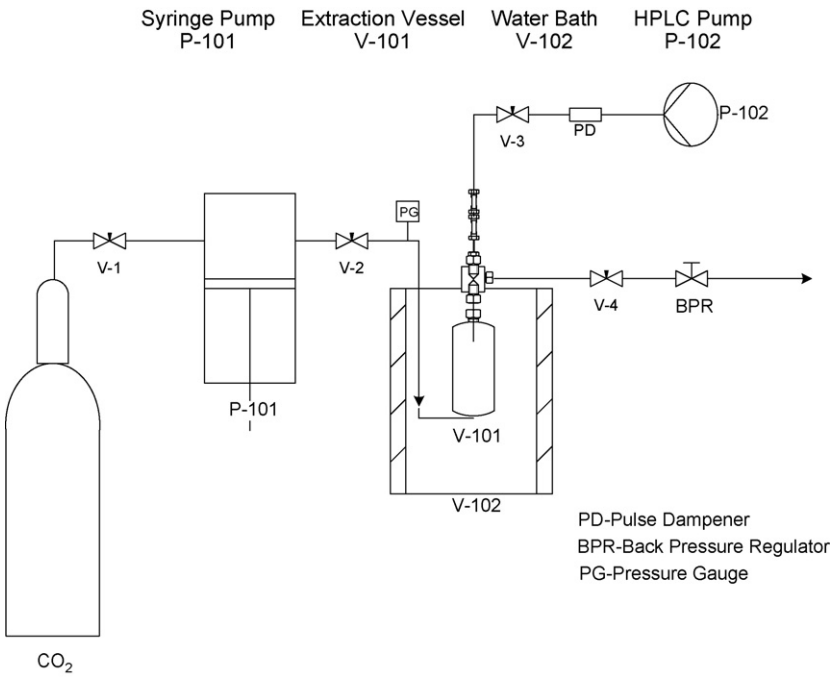

Fig. 1. SFEE piping and instrumentation diagram of semi-continuous process.

2.5. Semi-continuous SFEE process

The extraction of the emulsion and particle fabrication was carried out in a 40 mL cylindrical pressure vessel, placed in a temperature controlled water bath at 45 °C (Fig. 1). SC CO_2 was delivered by a syringe pump to the bottom of this vessel through a fitting containing a 0.5 μm stainless steel frit, which was further packed with glass wool. The emulsion was delivered countercurrently through a 100 μm capillary tube within a 1/16″ stainless steel high pressure tubing at the top of the vessel, allowing CO_2 to flow continually from the vessel. This system was backpressure regulated by a Tescom 6500 BPR to maintain a constant pressure of 8 MPa. The emulsion was sprayed into SC CO_2 at 0.4 mL/min (~7 mL total) and subjected to extraction for approximately 20 min. Once extraction was completed the vessel was depressurized over a 1 min span and an aqueous suspension of nanoparticles was collected from the pressure vessel.

2.6. Nanoparticle characterization

The particle size and size distribution was measured by photon correlation spectroscopy using a ZetaPlus™ Zeta Potential Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Zeta potential measurements were obtained by phase analysis light scattering using a Brookhaven Zeta PALS instrument. Each particle sample was suspended in Milli-Q H_2O and the averaged zeta potential and hydrodynamic diameter was calculated from five measurements performed on each sample. The procedures were repeated for three batches.

Particle morphology was studied utilizing transmission electron microscopy (TEM), performed by placing a drop of nanoparticle suspension (~1 mg/mL) on a copper grid. Excess liquid was removed with filter paper and a drop of 2% (w/v) NanoVan (methylamine vanadate, Nanoprobes) was added atop the nanoparticle suspension. After a 3 min incubation period the excess liquid was removed, allowing the grid to air dry. The nanoparticles were visualized using a Phillips 410 LS® transmission electron microscope (Phillips/FEI Inc., Briarcliff Manor, NY).

2.7. Nanoparticle plasmid loading and incorporation efficiency

Nanoparticle plasmid loading was acquired by centrifuging an aqueous suspension of particles and measuring the pDNA concentration of the supernatant using the Pico Green Assay (per manufacturer's protocol). Plasmid loading was calculated by subtracting the amount of pDNA removed from the supernatant from the total amount of pDNA initially sprayed in the emulsion formulation. The loading was expressed as μg pDNA/mg final product (nanoparticles) or % (w/w).

2.8. In vitro plasmid release

The nanoparticles (500 μg) were suspended in TE buffer (0.5 mL) and incubated at 37 °C on a shaker at 100 rpm. At selected time points, the nanoparticles were centrifuged at 12,000 rpm forming a pellet and the buffer supernatant analyzed for the amount (Pico Green Assay) of released plasmid DNA. At each time point, the nanoparticles were resuspended in fresh TE buffer.

2.9. Residual organic solvent

To determine the residual solvent content in SFEE processed nanoparticles, gas chromatography based headspace analysis was carried out using a Varian Chrompack CP 3380 with flame ionization detection (Koushik and Kompella, 2004). For this purpose, 1 mg of nanoparticles was placed in a 4 mL glass vial fitted with septa closure and dissolved in 500 μL methylene chloride. The sealed container was incubated at 60 °C for 10 min and 50 μL of headspace was injected onto the gas chromatography column. The column temperature was initially equilibrated to 40 °C for 1 min and then increased from 40 to 70 °C at 10 °C/min. Standard curves were generated by placing known amounts (in ppm) of ethyl acetate into methylene chloride preparations containing blank polymer (PLGA 85:15, 1 mg/sample). The peak area (PA) ratio response recorded was: ethyl acetate_{PA}/(ethyl acetate_{PA} + methylene chloride_{PA}). For this headspace GC analysis the limit of detection (LOD) was 50.5 ppm and the limit of quantitation (LOQ) was 168.2 ppm.

2.10. In vitro transfection

A549 cells were plated in a T-75 flask and grown to confluency. Cells were seeded into a 96-well plate at a seeding density 10,000 cells/well and grown to 60% confluency under culture conditions of either normoxia – 21% O_2 or, hypoxia – 1% O_2. 12 h prior to treatment, serum-free media for hypoxia was transferred to the tri-gas incubator. Serum-containing media was removed from the cells, washed once with PBS, and replaced with fresh serum-free media containing treatment. Cells were exposed for 4 h to the following treatment groups: control (no treatment), pFlt23K alone, pFlt23K + Lipofectamine™ (1 μg:3 μL ratio), and pFlt23K-PLGA polymeric nanoparticles. All treatments utilized the same amount of pFlt23K at 5 μg/well. At the end of the 4 h exposure the cells were washed once with PBS and fresh media replaced.

2.11. VEGF ELISA

Following the treatment at 96 h, supernatants were collected to estimate secreted VEGF using an enzyme linked immunosorbant assay (ELISA) according to manufacturer's recommendations (Research Diagnostics Inc., Flanders, NJ) (Ayalasomayajula and Kompella, 2003). All absorbance values were measured using a microtiter plate reader (Fischer Scientific, PA) using a test wavelength of 450 nm and a reference wavelength at 540 nm. The LOD for this assay was 20.5 pg/mL, while the LOQ was 68.4 pg/mL. All samples were above the LOQ for this assay.

2.12. Cytotoxicity

Effect of pFlt23K, pFlt23K + Lipofectamine™, and pFlt23K-PLGA polymeric nanoparticle (equivalent to 5 μg pDNA/well) treatment on cell cytotoxicity was assessed by the colorimetric MTT assay (Bandi and Kompella, 2001). The absorbance was measured using a reference wavelength of 540 nm using a microtiter plate reader. The percentage of viable cells with all tested treatments were calculated relative to untreated cells.

2.13. Statistical analysis

All data are expressed as mean ± standard deviation and comparison made using a one-way ANOVA with Tukey's *post hoc* analysis (SPSS for Windows; SPSS Science, Chicago, IL). Statistical significance was accepted at $p \leq 0.05$.

3. Results

3.1. Nanoparticle characterization

Governing factors for gene transfection in polymeric non-viral gene delivery systems include: pDNA loading, intracellular uptake of vector, and pDNA release from the vector. Therefore, the pDNA-PLGA nanoparticles were formulated and characterized for physical properties and subsequently *in vitro* gene transfection. Results of the physical characteristics of SFEE processed nanoparticles are summarized in Table 2.

TEM analysis of the SFEE nanoparticles revealed discrete particles that were spherical with smooth surfaces, ranging mainly between 80 and 280 nm in diameter (Fig. 2). Particles formed utilizing SFEE were a reflection of the initial emulsion droplet size and polydispersity. Initial droplet diameters of ~120 nm for both processed plasmid formulations yielded nanoparticles with hydrodynamic diameters of 322.3 nm (pFlt23K) and 148.9 nm (pEGFP), respectively. The ζ potential of the two SFEE-pDNA formulations was -17.7 ± 1 mV (pFlt23K) and -21.6 ± 6 mV (pEGFP), thus promoting stabilization of each nanoparticle formulation.

3.2. Nanoparticle plasmid loading and incorporation efficiency

The indirect method for plasmid loading analysis was justified due to incomplete plasmid extraction from SFEE-pDNA nanoparticles. An explanation for the incomplete extraction has been proposed as the precipitation of PLGA–PVA complex along with pDNA at the organic solvent–TE buffer interface, preventing complete pDNA partitioning into the aqueous phase (Prabha et al., 2002; Cohen et al., 2000). The loading analysis revealed 1.99% (w/w) loading (19.9 μg pFlt23K/mg nanoparticles) with an incorporation efficiency of 99.9%. While these loading amounts have been achieved by conventional evaporative methods (Prabha et al., 2002; Prabha and Labhasetwar, 2004), there have been no reports of an unmodified pDNA-PLGA nanoparticulate system with such incorporation efficiency.

A similar assessment of pEGFP-PLGA-nanoparticle loading was made to determine the versatility of the SFEE system. The 99.9% incorporation efficiency from the pFlt23K-PLGA experiments raises the possibility that SFEE is able to increase pDNA loading significantly. Indeed, the pEGFP loading was increased ~10 times to 19.69% (w/w) (197 μg/mg nanoparticles), with an associated incorporation efficiency of 98.5%.

3.3. In vitro plasmid release

The *in vitro* cumulative amount release (μg) of pFlt23K and pEGFP from SFEE nanoparticles is shown in Fig. 3. The release from both SFEE-pDNA nanoparticles was biphasic with a slight burst release of 7% in the pFlt23K (2%, w/w, loaded) and 0.5% in the pEGFP (20%, w/w, loaded) nanoparticles, followed by a slower sustained release.

3.4. Residual organic solvent

The headspace GC analysis revealed that SFEE plasmid loaded nanoparticles did not contain quantifiable residual ethyl acetate, being the first report of residual solvents levels for a PLGA gene delivery system. The summary of three SFEE experimental runs can be seen in Table 3, denoting the response was far below the limit of detection at 50.5 ppm.

3.5. VEGF ELISA

The VEGF ELISA data revealed that pFlt23K + Lipofectamine™ and pFlt23K-PLGA nanoparticles significantly reduced the amount of extracellular VEGF secreted ($p \leq 0.05$) under normoxic and hypoxic conditions (Fig. 4). The normoxic VEGF levels in pg/mg

Table 2
Physical characteristics of SFEE–PLGA (85:15) nanoparticles.

Plasmid	Polymer (%, w/w_{EA})	Diameter (nm)	Zeta potential (mV)	Theoretical loading (%, w/w)	Experimental loading (%, w/w)	Morphology
Placebo	3	285 ± 14	−18.2 ± 1	–	–	Spherical particles
pFlt23K	3	322 ± 45	−21.6 ± 6	2	1.99	Spherical particles
pEGFP	1	149 ± 13	−17.7 ± 1	20.0	19.7	Spherical particles

$n = 3$ experiments ± SD.

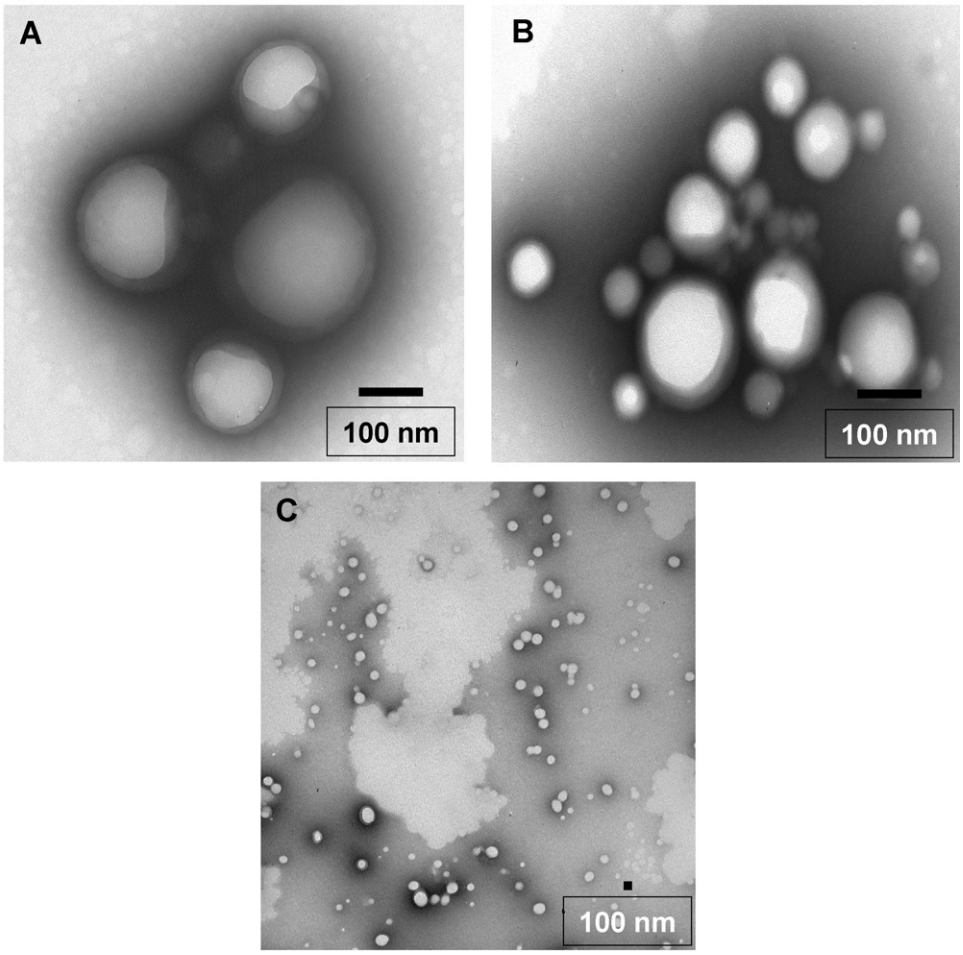

Fig. 2. Transmission electron micrographs of: (A) SFEE–PLGA (85:15)-placebo nanoparticles (69,000×), (B) pFlt23K-PLGA (2%, w/w, loaded) nanoparticles (82,000×), and (C) pEGFP-PLGA (20%, w/w, loaded) nanoparticles (54,800×).

total protein were 1489 ± 212 (control), 1726 ± 240 (pFlt23K alone), 396 ± 176 (pFlt23K + Lipofectamine™), and 848 ± 138 (pFlt23K-PLGA). The hypoxic VEGF levels in pg/mg total protein were 2206 ± 708 (control), 2513 ± 699 (pFlt23K alone), 572 ± 266 (pFlt23K + Lipofectamine™), and 1412 ± 266 (pFlt23K-PLGA). While both treatment groups were capable of reducing VEGF levels, the pFlt23K + Lipofectamine™ group had greater reduction.

3.6. Cytotoxicity

Following the same treatment and observation regime as in the VEGF experiment (under normoxic and hypoxic conditions), there was no significant cytotoxicity observed (Fig. 5). The amount of cell viability relative to control amounts under hypoxia were 98.6 ± 8.9% (pFlt23K alone), 96.2 ± 15.2%

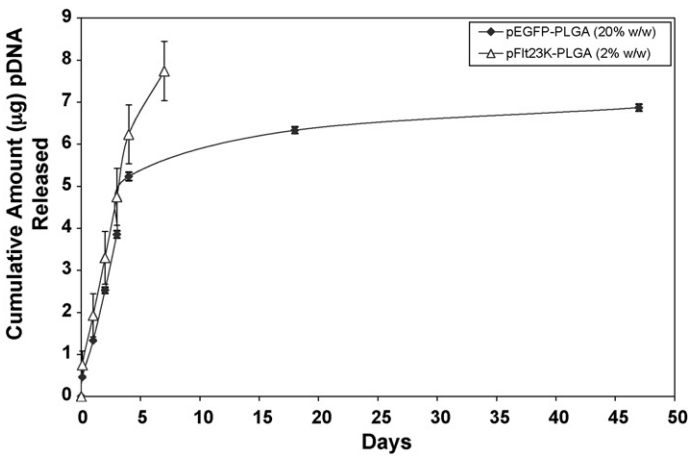

Fig. 3. *In vitro* cumulative amount released (μg) of plasmid DNA from pFlt23K-PLGA (2%, w/w, loaded) and pEGFP-PLGA (20%, w/w, loaded) nanoparticles. Data represented as mean ± SD ($n = 3$).

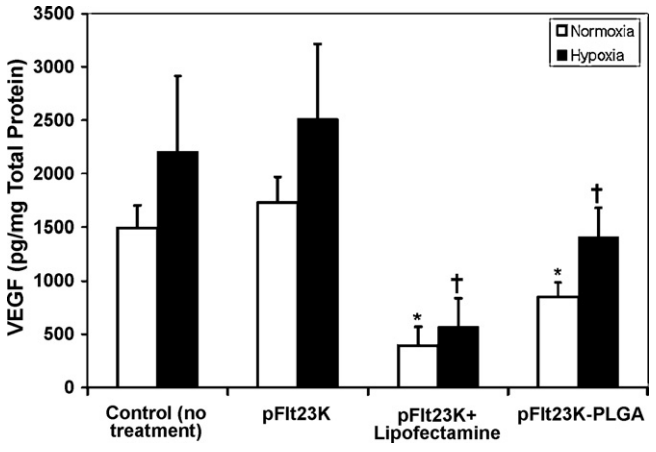

Fig. 4. VEGF secreted from A549 cells in response to various treatment groups. pFlt23K concentration used = 5 μg/well. The data is expressed as mean ± SD for $n = 8$; *significant difference from normoxia control, and †significant difference from hypoxia control ($p \leq 0.05$).

Table 3
Headspace GC residual solvent analysis of pFlt23K-PLGA–SFEE processed nanoparticles. Three experimental runs are summarized as mean ± SD.

Plasmid (2%, w/w)/batch	Polymer (3%, w/w_{EA})	Peak area ratio ($\times 10^{-5}$)	Ethyl acetate concentration (ppm)
pFlt23K/22606	PLGA 85:15	2.65	8.5
pFlt23K/3606r1	PLGA 85:15	0.55	1.7
pFlt23K/3606r2	PLGA 85:15	5.45	17.5
			9 ± 7 (<50, LOD)

(pFlt23K + Lipofectamine™), and 105 ± 7% (pFlt23K-PLGA). The cell viability under normoxia was 96.5 ± 14% (pFlt23K alone), 91.0 ± 23% (pFlt23K + Lipofectamine™), and 96.8 ± 9.4% (pFlt23K-PLGA).

4. Discussion

4.1. SFEE gene delivery nanoparticle formation

SFEE nanoparticle formation resulted from the SC CO_2 extraction of the organic solvent from the emulsion. Each emulsion droplet could be considered a "microreactor" in which supersaturation, particle nucleation, and particle growth occur as the organic phase is extracted (Shekunov et al., 2006). Once in contact with SC CO_2, mass transfer of the organic solvent has been proposed to occur via three parallel pathways: (1) direct mass transfer of the solvent due to the mixing of the SC CO_2 and the organic phase; (2) diffusion of the solvent into the aqueous phase, followed by subsequent mass transfer from this mixture into the SC CO_2; and (3) inverse flux of SC CO_2 into the emulsion droplets leading to expansion and extraction of the organic phase (Chattopadhyay et al., 2006a; Shekunov et al., 2006). Upon completed solvent extraction, the nanoparticles remain in the aqueous phase stabilized by the surfactant. The detailed quantitative mechanism of this process has not been defined, but the proposed mechanism for pDNA incorporation is illustrated in Fig. 6.

4.2. SFEE allows efficient plasmid loading

Upon fabrication of pFlt23K-PLGA nanoparticles, an attempt to extract the plasmid from the polymeric matrix was made by dissolving the particles in methylene chloride and then aqueous TE buffer was added for plasmid partitioning. A gel electrophoresis study showed incomplete extraction of the plasmid from the matrix or surfactant, which gave rise to use of indirect methods for loading measurement. Plasmid loading assessment via the Pico Green Assay revealed little apparent plasmid in the supernatant of the centrifuged nanoparticles, indicating extensive incorporation in and with the polymeric matrix. For experiments conducted to achieve 2% (w/w) loading (pFlt23K) and 20% (w/w) (pEGFP), plasmid loading levels reached 1.99% (w/w) and 19.69% (w/w), respectively. The corresponding incorporation efficiency was 99.9% and 98.5%, respectively.

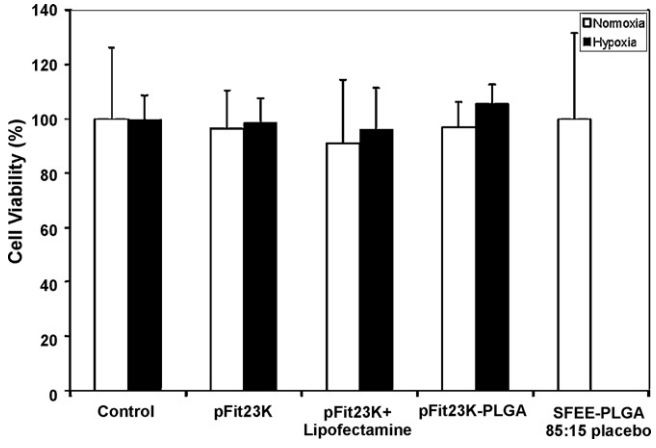

Fig. 5. MTT assay assessing the cytotoxicity of the various treatments in VEGF ELISA experiments. The data is expressed as mean ± SD for $n = 5$.

The loading assessment could be negatively effected due to the degradation of pDNA in carboxylic acid when SC CO_2 mixes with the outer aqueous phase. Investigators have shown that there is a pH decrease to 3 in an equilibrium water/CO_2 system (Tservistas et al., 2001; Ghenciu et al., 1998). This might not necessarily affect the pDNA contained within the emulsion droplet; however, it could have caused a surface charge reversal of the precipitating PLGA (Panyam et al., 2002) inducing electrostatic interactions with the pDNA, which could lead to increased exposure of pDNA at the surface of the matrix-type nanoparticles formed (Cohen et al., 2000). This leads the authors to more appropriately term the loading as incorporation rather than encapsulation.

4.3. In vitro plasmid release

The *in vitro* release profiles showed that the amount released from both pFlt23K-PLGA (2%, w/w, loaded) and pEGFP-PLGA (20%, w/w, loaded) nanoparticles are very similar (~5.5 μg) over the same period of time (4 days). One explanation for this could be surface associated pDNA, where release is primarily controlled by diffusion (Mahato, 2005). This might have caused the linear nature

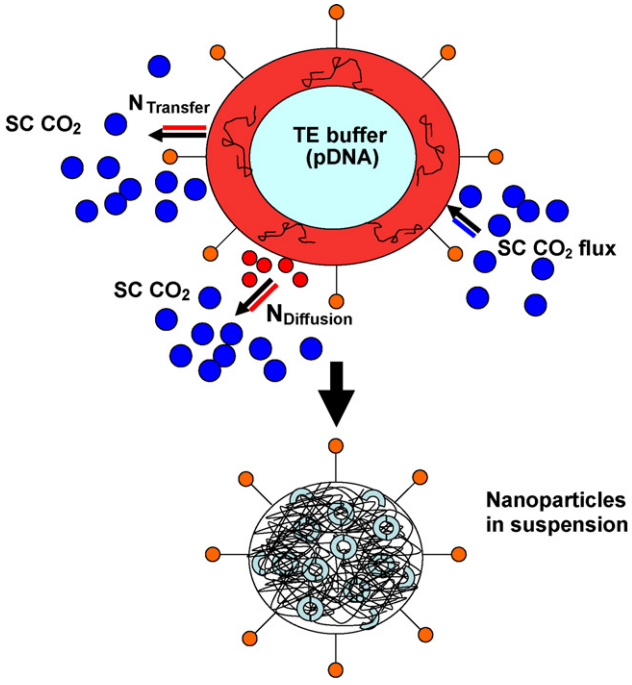

Fig. 6. Mechanism (proposed by Chattopadhyay et al. modified for plasmid DNA incorporation) of mass transfer in solvent extraction during SFEE processing. $N_{Transfer}$: direct mass transfer of solvent into SC CO_2; $N_{Diffusion}$: diffusion of solvent into emulsion continuous phase, subsequent mass transfer from this mixture into SC CO_2. SC CO_2 flux: SC CO_2 movement into the emulsion droplet leading to expansion of the organic phase.

of the release with limited burst effect, while other investigations experienced approximately 20–30% burst within 2–4 h, followed by a more sustained release (Cohen et al., 2000; Prabha et al., 2002; Prabha and Labhasetwar, 2004). The cumulative percent released from pFlt23K-PLGA nanoparticles at day 4 was ~60%, corresponding well with similar PLGA-pDNA gene delivery systems over the same time period (Cohen et al., 2000).

Interestingly, the cumulative percent release of plasmid from pEGFP-PLGA was approximately 10 times less than that of pFlt23K-PLGA nanoparticles over the same time period. However, plasmid loading was 10 times greater for pEGFP-PLGA, which might indicate a saturation point in the amount of surface associated pDNA. The release study for the pEGFP-PLGA nanoparticles was extended past the 4 days and after 7 days exhibited much less release than the initial period (Fig. 3), indicating a degradation controlled release (Wang et al., 1999). Additionally, the same report showed that increasing molecular weight (6–60 kDa) of PLGA 50:50 decreased the percent pDNA released fivefold over the same period of time. This could explain the lower amount released after the initial surface associated pDNA diffused from the nanoparticles, as the PLGA 85:15 utilized in this study had a molecular weight of 90 kDa.

The amount released from both SFEE nanoparticles was greater than most reports using similar release conditions and time periods (Wang et al., 1999; Cohen et al., 2000; Prabha et al., 2002, 2004). As explained earlier, this could be attributed to an increased amount of surface associated pDNA of the matrix-type system due to enhanced electrostatic attractions from the surface charge reversal of the precipitating PLGA in the emulsion by formation of carboxylic acid decreasing the aqueous pH.

4.4. SFEE allows efficient organic solvent removal

Chattopadhyay et al. (2006a) utilized a smaller extraction chamber (25 mL) and a flow rate of ~5 mL/min to accomplish extraction of ethyl acetate to a predefined level of approximately 40 ppm in 45–90 min in the particle suspension itself, not in the particles. The residual ethyl acetate values reported were from the batch experiments, where 10 mL of emulsion was placed into the extraction vessel. SFEE experiments in this study were conducted in a larger vessel (40 mL) at a slightly lower CO_2 flow rate (~4 mL/min) with introduction of emulsion at 0.4 mL/min. Gradually adding emulsion to the system enabled residual organic levels within the particles to reach undetectable levels within the total processing time. In this study, greater amounts of SC CO_2 interfaced with the emulsion droplets increasing mass transfer rates, thus reducing the ethyl acetate to undetectable levels in the particles (Table 3).

4.5. SFEE gene delivery nanoparticles reduce VEGF secretion without cytotoxicity

The efficacy of the pFlt23K intraceptor plasmid in reduction of hypoxia induced VEGF expression *in vitro* (human corneal epithelia cells) and *in vivo* (injury-induced corneal angiogenesis) has been previously demonstrated (Singh et al., 2005; Jani et al., 2007). Experiments were conducted to determine if the pFlt23K-PLGA nanoparticles were biologically efficacious in the reduction of VEGF levels in cancerous human lung alveolar epithelial cells. Results indicate that the amount of extracellular VEGF present in the cell supernatants exhibited a significant reduction ($p \leq 0.05$) with pFlt23K + Lipofectamine™ (positive control) and pFlt23K-PLGA nanoparticles. This confirms that the plasmid reached the intended target and subsequently expressed the gene.

Transfection with cationic lipid–pDNA complexes such as Lipofectamine was relatively more efficient; however, toxicity concerns and instability of these systems in the presence of serum can limit their effectiveness for *in vivo* applications (Liand and Huang, 2000; Nishikawa and Huang, 2001). If these systems were compared with serum present or *in vivo*, the results could be quite different. Additionally, cationic lipid–pDNA complexes have shown transient gene expression that decreases over sixfold during a period of 6 days (Prabha and Labhasetwar, 2004). pFlt23K-PLGA nanoparticles could result in sustained gene expression, benefiting in the treatment of chronic diseases that require low levels of protein expression at longer time intervals (Nishikawa and Huang, 2001).

The pFlt23K-PLGA nanoparticles exhibited no cytotoxicity under the VEGF study experimental conditions. The tetrazolium salt was converted to formazan crystals, indicating that the treatment groups did not affect the cells' mitochondrial function. Non-viral PLGA nanoparticulate systems have previously shown not to cause cellular toxicity (He et al., 2004).

5. Conclusion

PLGA 85:15 polymeric matrix-type nanoparticles incorporating pFlt23K and pEGFP plasmid DNA were successfully fabricated using SFEE. Electron microscopy images indicated that the nanoparticles formed were discrete spherical particles. The SCF processing enabled plasmid incorporation efficiencies to reach >98%, incorporated up to 19.7% (w/w) pDNA in PLGA, reduced residual organic solvent levels beneath detection, and released plasmid effectively from the nanoparticles. pFlt23K-PLGA nanoparticles were capable of *in vitro* transfection, reducing the amount of VEGF excreted from A549 cells under normoxic and hypoxic conditions, while not inducing cytotoxicity. *In vivo* studies are required to further determine the biological efficacy of SFEE fabricated pFlt23K-PLGA nanoparticles.

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