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Electrospray Production of Nanoparticles for Drug/Nucleic Acid Delivery

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1. Introduction

Nanomedicine – the application of nanotechnology to medicine – shows great potential to positively impact healthcare. Nanoparticles, including solid lipid nanoparticles, lipoplexes and polyplexes, can act as carriers to deliver the drugs or nucleic acid-based therapeutics that are particularly promising for advancing molecular and genetic medicine.

Many techniques have been developed to produce nanoparticles. Among them, electrospray has attracted recent research interest because it is an elegant and versatile way to make a broad array of nanoparticles. Electrospray is a technique that uses an electric field to disperse or break up a liquid. Compared with current technologies, such as bulk mixing, high pressure homogenization and double emulsion techniques, electrospray has three potential advantages. First, electrospray can generate monodisperse droplets whose size can vary from tens of nanometer to hundreds of micrometers, depending on the processing parameters. Secondly, it is a very gentle method. The free charge, induced by the electric field, only concentrates at the surface of the liquid, and does not significantly affect sensitive biomolecules such as DNA. Finally electrospray has the ability to generate structured micro/nanoparticles in a more controlled way with high drug/nucleic acid encapsulation efficiency.

Electrospray is a critical element of electrospray ionization mass-spectrometry, an analytical technique used to detect macromolecules that was developed by the 2002 chemistry Nobel Prize winner, Dr. John B. Fenn. (Fenn et al. 1989) Since then research has focused both on developing a fundamental understanding of the process as well as exploring potential applications of electrospray in fields ranging from the semiconductor industry to life science. During the past two decades, electrospray has been used to assist pyrolysis reactions and chemical vapor deposition processes, to produce inorganic particles including fine metal powder (Sn, Ag, Au, etc.), metal oxide particles (ZrO₂, TiO₂, etc.), ceramic particles (Si, SiO₂), and semiconductor quantum dots (CdSe, GaAs, etc.) (Jaworek 2007; Salata 2005). Various structured particles have been produced via electrospray, including

poly(methyl methacrylate)-pigment nanoparticles (Widiyandari et al. 2007), cocoa butter microcapsules containing a sugar solution or an oil-in-water emulsion (Loscertales et al. 2002; Bocanegra et al. 2005) and microbubble suspensions (Farook et al. 2007), to name a few. Electrospray has been used to deposit particle suspensions to form thin film (Jaworek & Sobczyk 2008, Jaworek 2010) or on-demand patterns, such as silica particle coatings on a quartz glass (Jaworek 2007; Salata 2005). Electrospray has been successfully applied in tissue engineering, for example, polymer materials including poly(lactide-co-glycolide) or poly(ethylene glycol) were electrospray-coated on biomedical implants (Kumbar et al. 2007). Combined with electrospinning, electrospray was used to fabricate smooth muscle cell integrated blood vessel constructs. (Stankusa et al. 2007) In the area of drug/nucleic acid delivery, many biological materials, such as DNA, proteins, and lipids have been electrosprayed without changing their biological activity (Pareta et al. 2005; Jayasinghe et al. 2005; Davies et al. 2005; Wu et al. 2009a, 2009b, 2010, 2011). Proteins, such as bovine serum albumin, have been encapsulated in biodegradable polymeric microcapsules (Pareta and Edirisinghe, 2006; Xie & Wang, 2006), and small molecule drugs, such as taxol and griseofulvin, have been encapsulated in polymeric microparticles for systemic or oral delivery. (Xie et al. 2006; Zhang et al, 2011)

2. Principles of electrospray

As illustrated in Figure 1a, in the standard electrospray configuration, a conducting liquid is slowly injected through a needle by a syringe pump. An electrical potential is applied to the needle to introduce free charge at the liquid surface. The free charge generates electric stress that causes the liquid to accelerate away from the needle. When the electrical potential rises to several kilovolts, the liquid meniscus at the needle opening develops into a conical shape, commonly called the Taylor cone. At the cone apex, where the free charge is highly concentrated, a liquid jet with high charge density is observed. Monodisperse particles are formed when the jet breaks into fine particles due to varicose or kink instabilities. (Cloupeau et al. 1994; Jaworek 2007; Salata 2005; Loscertales et al. 2002). Typically, the initial micron size droplets contain both solvent and a non-volatile solute and nanoparticles are produced as solvent evaporates from the high surface area aerosol.

Other electrospray configurations are also possible. Figure 1(b) shows a coaxial electrospray configuration, where two liquids are fed through the inner needle and the outer needle, respectively. This configuration is widely used to produce structured nanoparticles. Finally, since the flow rate in a single electrospray setup is always low ($\mu\text{L/hr}$ or mL/hr), multiplexed electrospray configurations have been developed to scale up production. (Deng et al, 2006) As shown in Figure 1(c), the single needle is replaced by a micro-nozzle array. All nozzles work simultaneously and produce nanoparticles with the liquid flow increased by orders of magnitude.

The electrospray modes and resulting droplet sizes d_D are controlled by the process parameters that include the applied voltage V , liquid flow rate Q , and liquid properties including electrical conductivity γ , surface tension σ , and liquid density ρ . (Cloupeau et al. 1994; Jaworek 2007; Salata 2005; Loscertales et al. 2002, Ganán-Calvo AM. 2004; Hartman et al. 1999; Basak et al. 2007) As illustrated in Figure 2, different electrospray modes are obtained as the applied voltage increases. (Cloupeau et al. 1994; Jaworek and Krupa 1999; Chen et al. 2005; Yurteri et al. 2010) These include dripping, micro-dripping, spindle,

Taylor cone-jet and multi-jet mode. Taylor cone-jet mode is the most common used electrospray mode, because it can produce highly monodisperse particles in a stable manner.

Fig. 1. Schematic diagrams of (a) standard electrospray (b) coaxial electrospray and (c) multiplexed electrospray. (d) Taylor cone-jet from standard electrospray (e) 91 nozzles used in multiplexed electrospray and (f) 91 Taylor cone-jets formed simultaneously. (Figures (e) and (f) are courtesy of Dr. Weiwei Deng).

Fig. 2. The electrospray modes change as the voltage increases. From left to right: dripping, microdripping, spindle, Taylor cone-jet and multi-jet.

In cone-jet mode, the droplet size scales with the liquid flow rate, and is inversely proportional to the liquid conductivity. Theoretically droplet sizes can be determined from the following scaling law, confirmed by many experiments (Jaworek 2007).

$$d_D = \alpha \left(\frac{Q^3 \varepsilon_0 \rho}{\pi^4 \sigma \gamma} \right)^{1/6} \quad (\alpha=2.9)$$

where ε_0 is the vacuum permittivity. In general, droplet size decreases with decreasing flow rate and increasing electrical conductivity of the liquid. By adjusting the flow rate and the liquid properties, droplets with the desired size can be produced in a well-controlled manner. Frequently, the initial solution contains both solvent and solute, and the final particle size (d_p) is related to the initial droplet size d_D by the following equation:

$$d_p = d_D \left(\frac{c \rho}{\rho_p} \right)^{1/3}$$

where c is the concentration of solute and ρ_p is the density of the particle.

3. Electrospray production of nanoparticles for drug/nucleic acid delivery

Our research group has been the first to explore electrospray as a means to produce solid lipid nanoparticles, lipoplexes and polyplexes for drug/nucleic acid delivery. Our results demonstrate the great potential of electrospray to produce nanoparticles in a well-controlled manner, and opens new avenues for the development of nanomedicine.

3.1 Solid lipid nanoparticles for hydrophobic drug delivery

3.1.1 Introduction

Solid lipid nanoparticles (SLNs) have been proposed as an alternative drug delivery system to traditional colloidal carriers, such as emulsions, liposomes and polymeric nanoparticles. As drug delivery vehicles, emulsions and liposomes are often limited by physical instability and low drug loading capacity. (Müller et al. 2000, 2004) Polymeric nanoparticles can exhibit cytotoxicity, and there are no industrialized production methods. SLNs may provide a drug delivery system that avoids many of these problems, and high pressure homogenization (HPH), the most popular preparation technique, is a reasonable method for large-scale production. Unfortunately, HPH requires molten lipids and high energy input. Thus, it is often unsuitable for use with temperature and shear sensitive biomolecules. Furthermore, as the mixtures cool, some drugs do not incorporate into the lipids but stick on the surface, resulting in burst drug release kinetics and low drug loading capacity. (Müller et al. 2000, 2004)

In our work we used electrospray to produce solid lipid nanoparticles. (Wu et al. 2009a, 2011) Cholesterol was chosen as a model since it is not only an important lipid but also a good model for lipophilic drugs. Similar to most lipophilic drugs, cholesterol has very low solubility in aqueous solutions, on the order of 1.8 µg/mL or 4.7 µM. (Haberland & Reynolds, 1973) Cholesterol has similar structure as the well-known anticancer drug Taxol®, and cholesterol (molecular weight = 387.6) is also a small molecule. In addition, the solubility of lipophilic drugs in lipids is much higher than in aqueous solutions, and, therefore, lipid nanoparticles, like the cholesterol nanoparticles discussed here, may also be useful as carriers to deliver lipophilic drugs.

3.1.2 Production of cholesterol nanoparticles

The standard electrospray configuration (Figure 1a) was used to produce cholesterol nanoparticles dispersed in aqueous media. Cholesterol powder (Sigma C3045) was dissolved in ethanol (Pharmco-Aaper E200GP) at a concentration of 2 mg/mL and then delivered to a 27 gauge needle by a syringe pump at a flow rate of 2 mL/h. A voltage of approximate 2.5 kV was applied to the needle and the observation of a stable Taylor cone-jet mode confirmed the proper conditions for generating fine droplets. The ethanol evaporated from the droplets, aided by heat input from a lamp, and the residual cholesterol particles were captured in 1X PBS supplemented with 1% Pluronic F68 placed in a grounded aluminum dish 5.5 cm below the needle tip. Figure 3 illustrates a typical size distribution measured for the cholesterol nanoparticle suspension. Highly monodisperse cholesterol nanoparticles were produced with sizes ~ 150 nm. The cholesterol concentration in the final solution was 180 ± 10 $\mu\text{g/mL}$ prior to sterile filtration and 150 ± 8 $\mu\text{g/mL}$ afterwards, i.e. up to 100 times higher than the solubility limit for cholesterol.

Fig. 3. A typical size distribution of cholesterol nanoparticles, in which D_p , mean, v was 140 nm and polydispersity was 0.107.

3.1.3 Bioavailability of cholesterol nanoparticles for NS0 cells

In this study, cholesterol itself was the bio-active ingredient, and NS0 cells were used to investigate the bioavailability of the cholesterol nanoparticles. While NS0 cells normally require serum as a cholesterol source, several research groups have demonstrated the growth of NS0 cells in protein free and chemically defined medium, using chemically defined cholesterol supplements. (Zhang & Robinson, 2005; Talley et al. 2005; Ojito et al. 2001) The auxotrophic nature of NS0 cells with respect to cholesterol makes this cell line a natural choice for characterizing the bioavailability of cholesterol nanoparticles produced by electrospray.

NS0 cells were continuously cultured in 250 mL shake flasks with 50 mL working volume in the basal medium supplemented with cholesterol nanoparticles or SyntheChol NS0 supplement (Sigma S5442, SyntheChol for short) at a cholesterol concentration level of 3.5 $\mu\text{g/mL}$. SyntheChol is a proprietary cholesterol supplement in liquid form and acts as the positive control. Figure 4 illustrates the viable cell density and cell viability as a function of

time. NS0 cells grew better with cholesterol nanoparticles than with SyntheChol reaching much higher peak cell densities: 5.28e6 cells/mL vs. 3.35e6 cells/mL.

Fig. 4. Growth of NS0 cells in media supplemented with 3.5 µg/mL of cholesterol nanoparticles or SyntheChol: viable cell density (a) and cell viability (b). The results are averages of triplicate runs.

Fig. 5. The effect of cholesterol concentration on (a) the growth, (b) the viability, and (c) the product titer of NS0 cells during fed-batch culture. Cholesterol nanoparticles or SyntheChol supplement were fed on day 0 and daily from day 2 till the end of culture at cholesterol concentration levels of 0.7 µg/mL, 1.75 µg/mL or 3.5 µg/mL. Error bars indicate one standard deviation of the results of triplicate runs. (ES CN: electrosprayed cholesterol nanoparticles)

The NS0 cell line is one of the important cell lines widely used by the pharmaceutical industry to produce therapeutic antibodies. We therefore investigated the effect of the cholesterol source on product titer by conducting a fed-batch culture of NS0 cells using electrosprayed cholesterol nanoparticles and the SyntheChol supplement at feed rates of 0.7, 1.75 and 3.5 $\mu\text{g/mL/day}$. Figure 5 summarizes the viable cell density, cell viability and normalized product titer (relative to the 1.75 $\mu\text{g/mL/day}$ SyntheChol supplement). At the lowest feed rate of 0.7 $\mu\text{g/mL/day}$, neither cholesterol nanoparticles nor SyntheChol supplement provided sufficient cholesterol to the cells and so cell growth was limited and the corresponding product titer was low (Figure 5c). When the feed rate of SyntheChol was increased, cell growth improved up to a feed rate of 1.75 $\mu\text{g/mL/day}$ but was compromised at feed rate of 3.5 $\mu\text{g/mL/day}$. The inhibitory effect of 3.5 $\mu\text{g/mL/day}$ feeding was presumably due to the compounds in SyntheChol that solubilize the cholesterol rather than to the presence of cholesterol. For cholesterol nanoparticles, both cell growth and viability improved at feed rates higher than 0.7 $\mu\text{g/mL/day}$. Compared to SyntheChol, culturing was extended from 8 days to 10 days. In general, the performance of cholesterol nanoparticles was better than that of SyntheChol supplementation at the same cholesterol feed rates. For both cholesterol nanoparticles and SyntheChol supplements, the highest product titer was achieved at a feed rate of 1.75 $\mu\text{g/mL/day}$ and the product titer with cholesterol nanoparticles was ~32% higher than that with SyntheChol.

3.1.4 Conclusions

Compared with conventional technologies, we have demonstrated that electrospray is an efficient way to produce solid lipid nanoparticles for drug delivery. In our application we used electrospray to produce highly monodisperse cholesterol nanoparticles, and investigated the bioavailability of the nanoparticles using the cholesterol auxotrophic NS0 cell line with SyntheChol as the positive control. We found that the cholesterol nanoparticles not only supported NS0 cell growth but also improved the titer of therapeutic antibody by 32% when compared with SyntheChol.

3.2 DNA/polycation polyplexes for gene delivery

3.2.1 Introduction

Viral and non-viral vectors have been developed to deliver nucleic acids to treat genetic and acquired disease. (Pack et al., 2005; Mastrobattista et al., 2006) Compared to viral vectors, non-viral vectors show lower immunogenicity, lower toxicity, better stability and lower cost. (Laporte et al., 2006; Kircheis, et al., 2001; Gebhart and Kabanov, 2001). Among the nonviral vectors, polyplexes and lipopolyplexes represent two major carrier systems. In this section, we focus on polyplexes for gene delivery, while in the following section we discuss the use of lipopolyplexes for oligonucleotides delivery.

Polyethylenimines (PEI) is a cationic polymer widely used to condense DNA and form polyplexes. Although PEI shows great promise for gene delivery both *in vitro* and *in vivo* (Godbey, et al., 1999; Neu et al., 2005), the delivery efficiency highly depends on the N/P ratio (the molar ratio of nitrogen in PEI to phosphate in DNA) and the preparation method. Bulk mixing (BM), the most commonly used method, is a simple and straightforward way to prepare DNA/PEI polyplexes. Unfortunately, the formation of the polyplexes is not well

controlled in bulk mixing, significantly affecting the particle size, structure, and, thus, the delivery efficiency. (Kircheis, et al., 2001; Gebhart and Kabanov, 2001) Alternative methods are needed to overcome this challenge.

Because electrospray is a gentle method that does not damage biomolecules it has attracted research interest in the realm of gene delivery. In some of the earliest work, enhanced green fluorescent protein (eGFP) plasmid was successfully delivered into African Green Monkey fibroblast cells (COS-1) using coaxial electrospray. (Chen et al. 2000) Since electrospray generates an aerosol, Davies et al. (2005) investigated gene delivery by exposing mice to naked pCIKLux aerosol generated via electrospray. Unfortunately, the *in vivo* delivery efficiency, 0.075%, was much lower than the 0.2% delivery efficiency achieved by Koshkina et al. (2003) when they nebulized pDNA/PEI polyplexes for pulmonary gene delivery. One possible reason for the low delivery efficiency of electrosprayed pCIKLux might be that the naked pDNA used is not stable in biological fluids and does not interact with cell membrane due to its negative charges. Our hypothesis was that pDNA/PEI polyplexes produced via electrospray might overcome limitations in the earlier studies. Thus, we used coaxial electrospray to achieve better control over the mixing of the DNA solution and the PEI solution, and thus to produce DNA/PEI polyplexes in a well-controlled manner. (Wu et al. 2010)

3.2.2 Coaxial electrospray DNA/PEI polyplexes

Coaxial electrospray was explored as a method to produce pDNA/PEI polyplexes using pGFP and pSEAP for qualitative and quantitative studies, respectively. The pDNAs were dispersed in OPTI-MEM medium at a concentration of 20 $\mu\text{g/mL}$. Branched 25 kDa PEI was dissolved in OPTI-MEM medium at concentrations of 17.2 $\mu\text{g/mL}$. In the coaxial electrospray setup, the pDNA solution flowed through the inner 27 gauge needle and the PEI solution flowed through the outer 20 gauge needle. The flow rates of the pDNA and PEI solutions were both set at 6mL/hr to yield an N/P ratio of 6.7. A positive voltage, typically 5~6 kV, was applied between the inner needle and a grounded copper ring electrode. The polyplexes were produced in the stable Taylor cone jet mode, captured in a grounded aluminum dish 5 cm below the needle tip, and used within 10 minutes.

The conventional bulk mixing method was also used to prepare pDNA/PEI polyplexes. Here PEI solution (17.2 $\mu\text{g/mL}$) was added into equal volume pDNA (20 $\mu\text{g/mL}$) solution to achieve an N/P ratio of 6.7. The resulting mixture was vortexed for a few seconds, incubated at room temperature for 10 minutes and used immediately.

3.2.3 pDNA damage analysis

To investigate the effects of coaxial electrospray on pDNA integrity, pGFP and pSEAP solutions were coaxial electrosprayed with OPTI-MEM medium containing no PEI. The flow rate for both pDNAs was set at 6mL/h, and the voltages were set at 5.0 kV for pGFP and 5.6 kV for pSEAP. Agarose gel electrophoresis was used to detect if the pDNA was damaged by coaxial electrospray. As illustrated in Figure 6, compared to the non-electrosprayed pDNA controls, little degradation of pDNA is observed. In addition, the ratio of supercoiled to open circular pDNA of the sprayed samples appears unchanged relative to the controls. Our

observations are consistent with those reported by Davies et al. (2005) and confirm that coaxial electrospray is a gentle but effective way to spray delicate bio-molecules.

Fig. 6. Gel electrophoresis shows that the pDNA is not damaged during the electrospray process. M: marker lane. C: control. E: electrosprayed pDNAs. Electrospray conditions: pGFP: flowrate of 6 mL/h and voltage of 5.0 kV; pSEAP: flowrate of 6 mL/h and voltage of 5.6 kV. OC: open circular. SC: supercoiled plasmid.

Fig. 7. Typical size distributions of the pDNA/PEI polyplexes prepared by coaxial electrospray and bulk mixing. The flow rate was 6 mL/h and the voltage was 5.0 kV for pGFP/PEI polyplexes and 5.6 kV for pSEAP/PEI polyplexes.

3.2.4 Size distribution of pDNA/PEI polyplexes

Dynamic light scattering was used to measure the size distribution of pGFP/PEI and pSEAP/PEI polyplexes prepared by coaxial electrospray, and bulk mixing. (Figure 7) The pDNA/PEI polyplexes prepared by either method are relatively monodisperse. The pGFP/PEI polyplexes prepared by coaxial electrospray had smaller particle size compared to those prepared by bulk mixing, while the sizes of the pSEAP/PEI polyplexes prepared by coaxial electrospray and bulk mixing were comparable. We note that the polyplexes prepared in this work were almost always larger than 400 nm because OPTI-MEM medium rather than NaCl solutions were used. OPTI-MEM medium was chosen to prepare polyplexes because it better mimics the *in vivo* situation than NaCl or PBS, and because its physical properties (electric conductivity and surface tension) are appropriate for the electrospray process. The downside is that OPTI-MEM medium contains reduced serum, and the adsorption of serum albumin and other negatively charged proteins enhances aggregation of polyplexes (Pack et al., 2005). Although the size distribution of each sample was measured within 10 minutes after preparation, the polyplexes were always larger than 300 nm suggesting that aggregates already formed. The adsorption of proteins to the polyplexes was also demonstrated by zeta potential measurements. The zeta potentials of all polyplexes samples were either close to zero or only slightly positive. Approaches such as grafting hydrophilic polymers, including polyethylene glycol (PEG) or polysaccharides, onto the PEI may help improve the stability of the polyplexes in serum (Pack et al., 2005; Laporte et al., 2006; Neu et al., 2001) and is one approach that could reduce aggregation.

3.2.5 pDNA/PEI polyplex transfection, DNA expression, and cell viability

The delivery efficiency of pGFP/PEI polyplexes produced by coaxial electrospray and bulk mixing was evaluated in NIH 3T3 cells and compared to Lipofectamine™ 2000, the positive control. As illustrated in Figure 8(a), at N/P ratio of 6.7, cells transfected with polyplexes produced by coaxial electrospray showed similar GFP expression as those transfected with Lipofectamine™ 2000, while cells transfected with polyplexes produced by bulk mixing showed much less GFP expression. No significant difference in cell viability was found among coaxial electrospray, BM and Lipofectamine™ 2000 ($p < 0.05$).

Since the evaluation of GFP expression is only qualitative, pSEAP was used to compare the delivery efficiency of polyplexes in a more quantitative way. As shown in Figure 9 (a), at N/P ratio of 6.7, cells transfected with the pSEAP/PEI polyplexes produced by coaxial electrospray gave 2.6 times higher SEAP expression than those produced by bulk mixing. Although cells transfected with Lipofectamine™ 2000 showed the highest SEAP expression, 3.3 times higher than bulk mixing, the cell viability was low because of the toxicity of this material. (Figure 9(b))

3.2.6 Conclusions

Both pGFP/PEI and pSEAP/PEI polyplexes were successfully produced using coaxial electrospray. The delivery efficiency of pDNA/PEI polyplexes was evaluated in NIH 3T3 cells. At N/P ratio of 6.7, polyplexes produced by coaxial electrospray were more effective at delivering genes to NIH 3T3 cells than those prepared by bulk mixing. Since coaxial

electrospray is an aerosol technique, it should be a useful way to deliver pDNA/PEI polyplexes directly to the lungs and achieve higher gene delivery efficiency than either electrospraying naked pDNA or nebulizing pDNA/PEI polyplexes produced by conventional methods.

Fig. 8. **(a)** The GFP expression in NIH 3T3 cells and **(b)** the cell viability measured 2 days post transfection. All polyplexes were produced at the N/P ratio of 6.7. For coaxial electrospray, the flow rate was 6mL/h and voltage was 5.0kV. (n=4)

Fig. 9. The pSEAP expression in NIH 3T3 **(a)** and the cell viability **(b)** measured 2 days post transfection. All polyplexes were produced at the N/P ratio of 6.7. For coaxial electrospray, the flow rate was 6 mL/h and voltage was 5.6 kV. (n=5, **: p<0.005)

3.3 Coaxial electrospray lipoplexes for oligodeoxynucleotides (ODN) delivery

3.3.1 Introduction

Lipoplexes, a major non-viral carrier system, have attracted a lot of research interest in the drug and nucleic acid delivery field. The phospholipid bilayer structure of lipoplexes provides great flexibility in encapsulating various drugs or nucleic acids, either within the hydrophobic bilayers or in the hydrophilic core. Many techniques, such as bulk mixing (Chrai et al. 2001; Elouahabi et al. 2005), the film method (Bangham method) (Fan et al. 2007; Otake et al. 2006) and alcohol dilution (Stano et al. 2004), have been developed to produce lipoplexes. However, non-uniform particle size, low encapsulation efficiency and multistep production process remain as major challenges. (Müller et al. 2000, 2004)

Antisense oligodeoxynucleotides (ODN) are short pieces of specially designed DNA. ODN has been widely investigated as a potential therapeutic agent against viral infections, cardiovascular inflammation, hematological malignancies, pulmonary diseases and cancer. (Chiu et al. 2006; Yang et al. 2004) ODN inhibits gene expression by hybridizing to specific mRNA sequences, which interferes with the target protein expression. For the preparation of ODN encapsulated lipoplexes, the ethanol dilution method is very popular (Stano et al. 2004; Jeffs et al. 2005). In this approach, the lipid mixture is first dissolved in ethanol and then mixed with the aqueous ODN solution. The final mixture is dialyzed against buffer solutions to remove ethanol and as the ethanol is removed ODN encapsulated lipoplexes are formed. The ethanol dilution method is clearly a batch production method, and batch to batch variation of the product can be an issue. In addition, multiple steps that may take days to complete are involved, and contamination of the final product can be an issue.

Our alternative approach was to use coaxial electrospray to produce ODN encapsulated lipoplexes, where the lipids/ethanol mixture initially surrounded the aqueous-ODN core. (Wu et al. 2009b) As the liquids leave the needles, the electrical field breaks the compound liquid stream into fine droplets. The large surface area of the droplets results in rapid ethanol evaporation and the ODN encapsulated lipoplexes are formed immediately. Compared to ethanol dilution, coaxial electrospray is a simple, one step and continuous production process. In addition, lipoplexes produced by coaxial electrospray can be either collected in buffer solutions for intravenous injection or used directly in pulmonary delivery.

3.3.2 Electrospray G3139 encapsulated lipoplexes

In our study, G3139 (Genasense or oblimerson sodium) was chosen as the model ODN. G3139 is an 18-mer ODN (5'-TCT CCC AGC GTG CGC CAT-3'). It is specially designed to bind the first six codons of the human Bcl-2 mRNA and thus inhibit Bcl-2 expression, and may provide a way to decrease the resistance of tumor cells to chemotherapy. (Chiu et al. 2006) To prepare G3139 encapsulated lipoplexes, G3139 was dispersed in 1X PBS solution fed through the inner 27 gauge needle. The lipid mixture (DC-Cholesterol: EggPC:DSPE-PEG=30:68:2 molar ratio) was dissolved in ethanol and fed through the outer 20G needle. A positive voltage, typically ~3 kV, was applied between the inner needle and a grounded copper ring electrode to break the liquid into fine droplets. As the ethanol evaporates the lipoplexes are formed and then captured in a grounded aluminum dish 10 cm below the needle tip containing 15 mL PBS solution.

3.3.3 Size distribution and zeta potential of G3139 encapsulated lipoplexes

The size distribution and surface charge of G3139 encapsulated lipoplexes prepared by coaxial electrospray depend on the concentrations and flow rates of G3139 solution and lipid mixtures. By adjusting operation parameters, the size of lipoplexes can vary between 100 nm and 2500 nm and the surface charge of lipoplexes can vary between -16 mV and +20 mV. (Wu et al. 2009b) The best balance between lipoplex productivity, size and surface charge, was achieved by setting the flowrates of the G3139 solution and the lipid mixture to 1.2 mL/h, the concentration of the G3139 solution to 0.5 mg/mL and the concentration of the lipid mixture to 10 mg/mL. Under these conditions Figure 10 shows that the lipoplex average mean diameter by volume was 190 ± 39 nm. The corresponding zeta potential was $+4.5 \pm 0.43$ mV and the final lipid/G3139 ratio was 20.

Fig. 10. A typical size distribution of G3139 encapsulated lipoplexes. The average mean diameter by volume was 190 ± 39 nm.

3.3.4 Structure of G3139 encapsulated lipoplexes

Small angle neutron scattering (SANS) and cryo-transmission electron microscopy (cryo-TEM) were used to characterize the structures of G3139 encapsulated lipoplexes. Figure 11 illustrates typical SANS spectra for lipoplexes made by coaxial electrospray and by an ethanol dilution method.

For the sample prepared by the ethanol dilution method, a Bragg Peak was observed, indicating the multi-lamellar structure of the lipoplexes, while the Bragg Peak was absent in lipoplexes produced by coaxial electrospray. From the position of the Bragg Peak we determined the inter-lamellar spacing d using the relation $d = 2\pi/q$, where $q = (4\pi/\lambda)\sin(\theta/2)$ is the momentum transfer vector, λ is the neutron wavelength, and θ is the scattering angle. When we fit the peak for the lipoplexes using a Gaussian function, we found a center-to-center lamellar spacing of 6.6 ± 0.2 nm. The cryo-TEM images support the SANS results by showing that most electrosprayed lipoplexes have a uni-lamellar structure, while lipoplexes prepared by ethanol dilution have onion like multi-lamellar structure with center to center inter-lamella distance measured from the picture of about 7-10 nm. The difference in the structure of lipoplexes produced by these two methods may reflect the removal rate of ethanol from the solution. In coaxial electrospray, the liquids break into tiny droplets, and

the ethanol evaporates in less than 1 s due to the large surface area of the fine droplets. Thus, the lipids and G3139 may be “locked” and do not have time to rearrange and form the multi-lamellar structure. In contrast, the dialysis step in the ethanol dilution method is a very slow process, usually requiring about 24 h to remove ethanol from the solution, and therefore the lipids and G3139 have enough time to form the more complex multi-lamellar structure.

Fig. 11. Typical SANS spectra and cryo-TEM images show that the electro sprayed lipoplexes have unilamellar structure, while those prepared by ethanol dilution have a multi-lamellar structure. The error bars on the SANS data represent $\pm$ one standard deviation and are generally smaller than the symbol size. (Scale bar: 100nm)

3.3.5 Bcl-2 down regulation

The bioactivity of G3139 encapsulated lipoplexes was evaluated in K562 cells (chronic myelogenous leukemia cell line). In addition to non-targeted G3139 encapsulated lipoplexes, transferrin (Tf) conjugated G3139 encapsulated lipoplexes were also used in this study. Chiu et al. (2006) reported that transferrin (Tf) conjugated lipoplexes had targeting ability because they could binding to the transferrin receptor (TfR), a transmembrane glycoprotein over expressed on cancer and leukemia cells. Thus, transferrin conjugated lipoplexes provided better down regulation of Bcl-2 in K562 cells and were more effective for *in vivo* applications. K562 cells were treated with both non-targeted and Tf-targeted G3139 encapsulated lipoplexes at G3139 concentration of 1 μ M. Bcl-2 expression were measured by western blotting 48 h post transfection. Figure 12 shows that the Bcl-2 expression was decreased by up to $\sim$ 55% when cells were transfected with Tf-targeted G3139 encapsulated lipoplexes, compared to $\sim$ 40% by non-targeted G3139 encapsulated lipoplexes and $\sim$ 15% by free G3139.

Fig. 12. A representative western blotting of Bcl-2 expression in K562 cells. LP: non-targeted G3139 encapsulated lipoplexes; Tf-LP: Tf-targeted G3139 encapsulated lipoplexes. The concentration of G3139 was 1 μ M. (n=3, *: $p < 0.05$, **: $p < 0.01$.)

3.3.6 Cellular uptake of ODN encapsulated lipoplexes

K562 cells were transfected by free FAM-ODN, non-targeted FAM-ODN encapsulated lipoplexes and Tf-targeted FAM-ODN encapsulated lipoplexes at FAM-ODN concentration level of 1 μ M. Flow cytometry and confocal microscopy were used to investigate the cellular uptake of FAM-ODN encapsulated lipoplexes 4 h post transfection. As shown on Figure 13 the cellular uptake of FAM-ODN delivered by lipoplexes, particularly Tf-targeted lipoplexes, was much more efficient compared to cells treated with free FAM-ODN. Compared to cells transfected with non-targeted FAM-ODN encapsulated lipoplexes, those treated with Tf-targeted FAM-ODN encapsulated lipoplexes had ~40% higher fluorescence signal, indicating that transferrin improved the interaction between the lipoplexes and the K562 cells and facilitated the cellular uptake of lipoplexes, and thus more efficient Bcl-2 down-regulation was observed. For future *in vivo* applications Tf-targeted lipoplexes might also be more effective targeting cancer cells and minimize the rapid clearance by the reticuloendothelial system, or side effects, such as nonspecific cytokine production.

Fig. 13. **(a)** Flow cytometry and **(b)** confocal microscopy images showed the cellular uptake of FAM-ODN by K562 cells. K562 cells were treated with free FAM-ODN, non-targeted FAM-ODN encapsulated lipoplexes (LP) and Tf-targeted FAM-ODN encapsulated lipoplexes (Tf-LP) at FAM-ODN concentration of 1 μ M. Control: cells cultured in medium with no transfection. DIC: differential interference contrast.

3.3.7 Conclusions

We developed a coaxial electrospray process to produce oligonucleotide encapsulated lipoplexes for nucleic acid delivery. The lipoplexes produced by coaxial electrospray can either be collected for intravenous injection or delivered as aerosol for inhalation therapy. This method allows for better control over the way in which the lipid and aqueous phases are mixed. Compared with the standard ethanol dilution technique, coaxial electrospray is a simple, one step, continuous process that significantly reduces the time and effort required to produce the lipoplexes. By adjusting operating parameters, such as flow rates and liquid concentrations, monodisperse lipoplexes with different size and surface charge can be easily produced to meet various application needs. In this work, G3139 encapsulated lipoplexes were successfully produced via coaxial electrospray with diameter of ~190nm and zeta potential of ~+4.5 mV. Due to fast ethanol removal, the lipoplexes produced by coaxial electrospray showed unilamellar structure compared to the multi-lamellar structure of lipoplexes produced by ethanol dilution method. Transferrin was successfully conjugated to the G3139 encapsulated lipoplexes. Flow cytometry and confocal microscopy analysis showed that transferrin provided targeting ability for the lipoplexes, which greatly improved the cellular uptake of lipoplexes. Compared to ~40% Bcl-2 protein down-regulation observed by non-targeted G3139 encapsulated lipoplexes, Tf-targeted lipoplexes was more efficiently delivered to K562 cells and down regulated the Bcl-2 protein expression by ~55%.

4. Summary

To summarize, we have demonstrated the great potential of electrospray to produce nanoparticles for a variety of drug/nucleic acid delivery applications, including solid lipid nanoparticles for hydrophobic drug delivery, as well as polyplexes and lipoplexes for nucleic acid delivery. We hope this review can stimulate further development and utilization of electrospray in nanobiotechnology.

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