Structural characterization of novel micro- and nano-scale non-viral DNA delivery systems for cutaneous gene therapy

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ABSTRACT

The structural and physicochemical properties of novel dicationic lipid-based DNA complexes have been investigated as micro/nano-scale self assembling delivery systems for cutaneous gene therapy. *In vitro* transfection efficiency (TE) and cutaneous delivery was dependent on the length of the spacer between the two positively charged head groups. AFM, particle size and ζ potential analysis indicated that the DNA/gemini (with or without DOPE) complexes were generally in the range of 100-200 nm and 40-50mV. Topical gemini-liposomal formulations contained two populations of particles (100-200 nm and 2-5μm) and the particle size in the gemini-nanoemulsion formulation was 5-10 nm. SAXS measurements indicate that gemini surfactants with shorter spacers have greater ability to induce polymorphic structures in the generally lamellar complexes, and exhibit greater transfection activity and cutaneous delivery.

1 INTRODUCTION

The objective in gene therapy is the introduction of a missing or defective gene into the cell nucleus where the encoded protein can be expressed. During the transfection process the DNA crosses several barriers such as the cellular, lysosomal, and nuclear membranes prior to expression. In the absence of a suitable carrier/vector/delivery system DNA is not taken up into cells therefore protein is not expressed. Viral vectors have been shown to increase DNA delivery, however due to safety and production concerns better systems are needed [1]. Non-viral approaches offer many advantages including absence of viral components and lack of immunogenicity; they are less expensive, easily manufactured and can be readily altered to form different combinations depending on the intended treatment. Various types of cationic supramolecular assemblies of DNA have been developed, however, further improvements are still needed to improve efficiency.

Our main goal is to develop micro/nano-scale self-assembling delivery vehicles for cutaneous gene therapy. The objective of this study was to characterize the structural and physicochemical properties of novel dicationic lipid-based DNA complexes by atomic force microscopy (AFM), small-angle x-ray scattering (SAXS), zeta potential and particle size analysis in order to determine the optimum parameters required for cellular transfection and cutaneous transfer.

2 MATERIALS AND METHODS

A series of cationic lipid-DNA complexes based on dicationic (gemini) surfactants and other lipids of various compositions were constructed. Transfection mixtures consisting of plasmid – gemini surfactant complexes, (PGs – 1:10 plasmid:gemini surfactant charge ratio) and plasmid – gemini surfactant – helper lipid vesicles (PGLs – with 1mM dioleoyl-phosphatidylethanolamine (DOPE) as helper lipid) were prepared, by first complexing the DNA with the cationic surfactant, followed by addition of the helper lipid. More complex topical liposome (composition: DOPE 10 mg/mL, dipalmitoylphosphatidylcholine (DPPC) 10 mg/mL, gemini 16-3-16 surfactant 10 mg/mL, and diethylene glycol monoethyl ether 25 mg/mL, containing 25 μg of plasmid/50μL) and nanoemulsion formulations (composition: PEG-8 caprylic/capric glycerides 200 mg/mL, polyglyceryl-3-isostearate 200 mg/mL, octyldodecyl myristate 400 mg/mL and the gemini 16-3-16 surfactant 10mg/mL) were made. All lipids for the complexes, liposomes and nanoemulsion were obtained from Avanti Polar Lipids and Gattefosse.

Murine keratinocytes (PAM212 cell line) at $5x10^4$ cells/well were grown to 60-70% confluency. The cells were transfected with PGs or PGLs containing 0.2 μg plasmid/well. The plates were incubated for 5 hours at 37°C in a CO_2 incubator. The supernatants were collected at 24 hours. The expressed protein (murine interferon γ) was determined by ELISA.

AFM measurements were made using a Molecular Imaging Inc. PicoSPM instrument, in MAC-mode, using MI MAC cantilever Type II (K=1.2-5.5N/m). The DNA, PGs and PGLs, 10μl each were spread on the surface of freshly cleaved mica, and incubated for 30 seconds to 15 minutes at room temperature. The excess formulation was removed with lint free absorbent tissue, and the mica surface dried with N_2. A 4x4 μm or 35x35 μm surface area was scanned.

SAXS measurements were made using beamline X21 at the National Synchrotron Light Source at Brookhaven National Laboratory. The measurements were performed with 12KeV x-rays and the data covered a q-range from $0.008Å^{-1}$ to $0.5Å^{-1}$. Samples

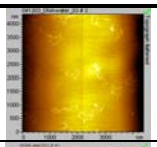
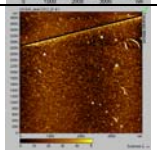
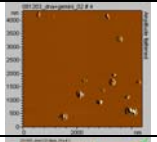
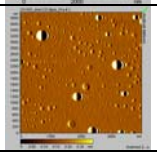
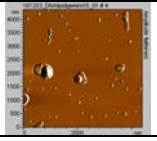
System	AFM		Light Scattering		SAXS		
	Size (nm)	Image	Size* (nm)	ζ* (mV)	q ($Å^{-1}$)	d (Å)	Structure
DNA	100-1000		50-100	-47 ± 15	-	-	No profile observed
12-3-12/DNA	-		130 ± 30	15 ± 2	0.142	44	Single peak
16-3-16/DNA	~100 uniform		220 ± 70	58 ± 1	0.130	48	Single Peak
12-3-12/DNA/DOPE	-		140 ± 20	41 ± 5	0.078 0.110 0.222	57	Lamellar with possible second phase
16-3-16/DNA/DOPE	100-300		210 ± 30	44 ± 4	0.100 0.198	63	Lamellar

Table 1: Properties of transfection formulations for *in vitro* studies (*average of 3 separate samples, 3 repeats each)

were loaded into 1.5 mm capillaries and the scattering pattern was recorded using a 13cm Mar CCD detector (Mar USA, Evanston, IL), at 1.26m (calibrated with the scattering pattern of silver behenate) downstream of the sample. All spectra were processed to remove background contributions by subtracting the scattering profile obtained for a water-filled capillary.

Particle size and Zeta potential (ζ) measurements were made using a Malvern Zetasizer NanoZS instrument and data were processed using the Malvern DTS software.

3 RESULTS

We have tested eight different gemini surfactants (plasmid DNA–gemini surfactant–DOPE; cationic charge ratio 1:10) to determine the effect of head group spacer length and alkyl chain length on their transfection ability *in vitro*. These studies indicated that the transfection efficiency (TE) and cutaneous absorption was dependent on the length of the spacer between the two positively charged head groups, with C3 spacer showing the highest activity [2]. We have previously shown that the transfection efficiencies for the gemini surfactants are correlated to other physical properties (such as the head group area, critical micelle concentration, etc.) that depend upon the size and/or nature of the spacer group. In this work, the

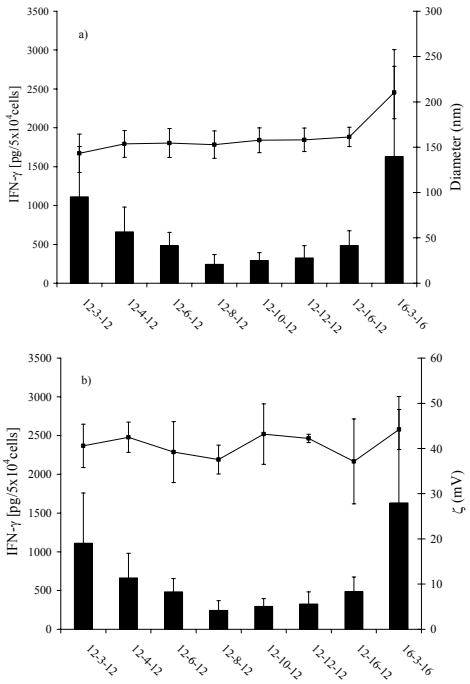


Figure 1: Correlation of transfection efficiency shown as IFNγ expressed (bars) with a) particle size and b) zeta potential of the DNA-gemini-DOPE (PGL) complexes.

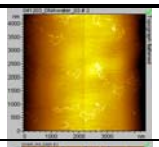
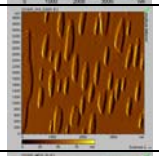
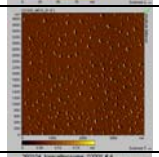
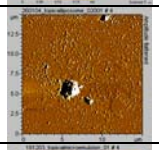
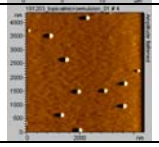
System	AFM		Light Scattering		SAXS		
	Size (nm)	Image	Size* (nm)	ζ* (mV)	q ($Å^{-1}$)	d (Å)	Structure
DNA	100-1000		50-100	-47 ± 15	-	-	No profile observed
16-3-16 FL	100-1000		250 5000	61	0.120	52	Broad peak
16-3-16 ME	50-100		3	-	~0.03	~210	Broad peak associated with interdrop correlation
16-3-16 FL / DNA	100 2000-3000		160 450	52	0.038 0.064 0.094 0.186	67	Lamellar with possible second phase
16-3-16 ME / DNA	100-300		5	-	~0.03	~210	Broad peak associated with interdrop correlation

Table 2: Properties of topical formulations for *in vivo* studies (*average values)

AFM and light scattering analysis indicated that the DNA/gemini complexes were generally in the range of 100-200 nm, in agreement with previous results [3]. While the variations in the size of the plasmid/gemini surfactant/DOPE complexes are small, specific correlation was not observed between size (Figure 1a) and transfection efficiency, as a function of spacer group. Interestingly, as can be seen in Table 1 and Figure 1 by comparing data for the 12-3-12 and 16-3-16 surfactants, smaller particle size was not a requirement for increased transfection efficiency.

Similarly no correlation is observed between the zeta potential (ζ) and transfection efficiency (Figure 1b); however it can be seen that ζ in all cases is > 30 mV, indicating that not only do the complexes possess the necessary positive surface charge needed for transfection, but also that the complexes have sufficient surface charge to remain, generally, stable in solution. The effect of variation in the spacer group, at a fixed alkyl tail length, on the structure of the PGL systems determined from SAXS is more complex. Figure 2 illustrates the scattering profiles for the PG (Figure 2a) and PGL (Figure 2b) systems. For the PG systems the position of the scattering peak correlates, generally, with other properties of the surfactant specifically related to the size of the surfactant head group. Contrary to the PGL systems described below,

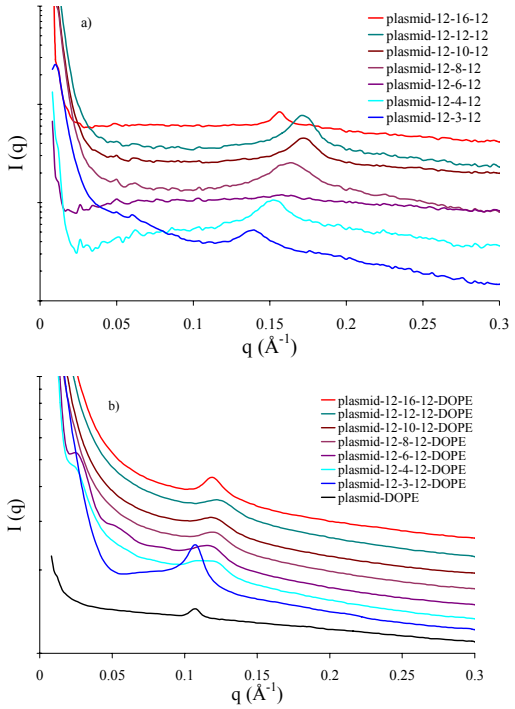


Figure 2: SAXS profiles of a) DNA-gemini surfactant complexes and b) DNA-gemini-DOPE complexes

the DNA-gemini systems did not appear to form other polymorphic structures and did not transfect cells.

For the PGL systems, the 12-3-12 surfactant exhibits a lamellar morphology; however, a weak scattering peak is also evident at q = 0.096 $Å^{-1}$. Similar results are obtained for the 12-4-12 and 12-6-12 PGL systems, and are indicative of the presence of additional phases; however the identities of these phases are not yet known. It appears that these gemini surfactants form mixed polymorphic systems in the presence of DNA and DOPE and have the ability to induce polymorphic structures other than hexagonal in the predominantly lamellar PGL systems, which may facilitate the eventual release of the DNA resulting in increased transfection. This was specifically observed for the surfactants having short spacer groups. This is an interesting observation in the light of previous reports where a hexagonal structure thought by many to be more efficient at transferring DNA to cells [4, 5]. Plasmid-DOPE complexes without any gemini surfactant show a typical hexagonal profile (q = 0.107 and 0.185 $Å^{-1}$; Figure 2b); however these complexes do not have the ability to transfect PAM212 cells.

The topical liposomal formulation prepared with 16-3-16 surfactant contained two populations of particles (100-200 nm and larger 2-5μm particles; as seen by both light scattering and AFM) and the particle size in the nano-emulsion formulation was 5-10 nm (Table 2). Additionally, the scattering profiles obtained for the two systems are markedly different. Profiles obtained for the blank liposomal and both blank and DNA-containing microemulsion formulations were featureless, exhibiting only broad peaks. The addition of DNA to the liposomal formulation appears to stabilize the structure, as shown in Figure 3. Subtraction of the profile obtained for the blank formulation results in features resulting from the complexation of the DNA with the liposomes, and shows both a lamellar phase (peaks 3 and 4) as well as other possible phases (peaks 1 and 2). The *in vivo* efficiency [2] of topical liposomal and nanoemulsion formulations was not dependent on size, however, structural characteristics such as the presence of both lamellar and other polymorphic (possibly cubic phases) appear to play a greater role (Table 2 and Figure 3). Further investigations regarding the differences between the mechanism for transfection in these systems are necessary and are currently under way.

4 CONCLUSION

Successful transfection is a complex process, dependent on many factors which are still not fully understood. We have demonstrated here that particle size variation of DNA-gemini-DOPE complexes may not be a significant factor in *in vitro* transfection or *in vivo* cutaneous delivery below a certain limit, possibly below 200 nm. However, we found that the most important factor for both *in vitro* and *in vivo* efficacy is the ability of cationic complexes to form polymorphic structures [6].

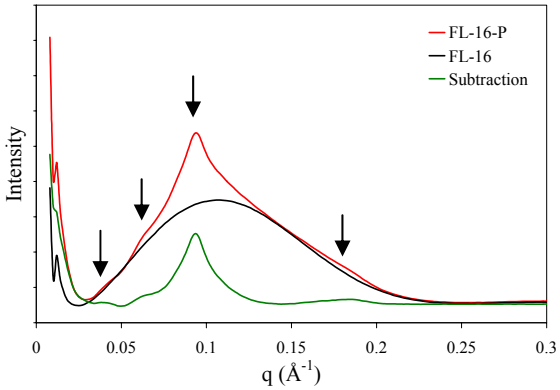


Figure 3: SAXS profiles for FL-16 topical formulations with and without added plasmid and a plot of the FL-16 with plasmid corrected for the blank formulation. Arrows left-to-right indicate peaks 1-4.

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