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Development and Characterization of a Cationic Emulsion Formulation as a Potential pDNA Carrier System

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The development of efficient and stable carriers for the delivery of DNA to the body is becoming an increasingly important issue in the fields of gene therapy and vaccination. The present study was designed to prepare an emulsion-based gene delivery system. Oil-in-water emulsions containing cetyltrimethylammonium bromide (CTAB) as a cationic surfactant and Pluronic F-68 as a nonionic co-surfactant were formulated and their physical characteristics were investigated. The cationic emulsion, referred to as B13d, containing a liquid crystal phase, was found to be stable and suitable for parenteral application. Complex formation between pGL3 plasmid DNA and B13d emulsion achieved at the pDNA:CTAB ratio of 1:2.74 (μg/nmol) was demonstrated using the gel retardation and SYBR Green I displacement assays. Particle sizes of the freshly prepared empty and pDNA-associated B13d emulsions were about 350 nm. Zeta potentials of empty and pDNA loaded emulsions were +45.3 mV and +43.7 mV, respectively. The sensitivity of pDNA/emulsion complexes to endonuclease digestion was determined and complexes were found to be highly effective for protecting pDNA from DNase I attack. The final in vitro stability analysis carried out in the presence of human serum revealed that the super-coiled pDNA was observable even after 24 h of incubation. The physical characteristics and serum resistant properties of the complexes suggest that B13d emulsion could be an efficient pDNA carrier system for gene and/or immunogene delivery.

Key Words: Gene delivery, Cationic emulsions, Cetyltrimethylammonium bromide (CTAB), Plasmid DNA.

Introduction

The production of therapeutic proteins by the patient's cells may provide a safe and cost-effective treatment for a variety of genetic and acquired diseases. The transfer of genes into eukaryotic cells for therapeutic

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purposes requires the use of a stable and effective carrier system to accomplish optimal and prolonged gene expression. Research in the field of gene therapy has focused on the development of suitable gene carrier systems that enable the efficient transfection of cells in vivo. Many different viral and nonviral systems are currently under investigation[1]. Viral systems are highly efficient in transfection; however, the serious side effects of viral vectors related to host immune and inflammatory responses limit their use in clinical applications[2−3]. On the other hand, nonviral systems in which plasmid DNA or oligonucleotide is complexed with cationic liposomes[4], polymers[5], peptides[6] or other carriers[7] provide an attractive alternative route for gene delivery. Nevertheless, the efficiency of transfection with nonviral systems is relatively low and each protocol has its distinct problem regarding stability, efficacy or toxicity. Consequently, studies for the optimization of existing systems and for the development of novel formulations are ongoing.

Recently alternative recipes involving the association of plasmid DNA or oligonucleotides with colloidal carrier systems, such as emulsions[8−11], microspheres[12] and nanocapsules[13], have drawn attention for gene and immunogene delivery. In particular, there has been increasing interest in emulsion-mediated transfection, and several reports demonstrating the potential of positively charged emulsion particles as a new delivery system for gene therapy have been published over the past few years. For example, Liu et al. prepared oil-in-water emulsions consisting of castor oil, DOPE, DC-Chol and 4 different types of surfactants including Tween, Span, Brij and Pluronic copolymers as co-emulsifiers and studied the effect of nonionic surfactants on the particle size, transfection activity and serum sensitivity of DNA/emulsion complexes. It was observed that the inclusion of nonionic surfactants in the formulation resulted in profound effects on the features of emulsions. The transfection activity of each formulation varied significantly, and Tween, Brij 72, Pluronic F68 and F127 containing emulsions demonstrated increased activity in a transfection experiment using mouse BL-6 cells in the presence of 20% serum[8]. In another study, Teixeira and co-workers used oligothymidylates of various lengths that were incorporated into submicron cationic emulsions containing lecithin, medium-chain triglycerides, poloxamer 188 and various concentrations of stearylamine. When the characteristics of the complexes were investigated in vitro, the formulation of oligothymidylates in a cationic emulsion containing 0.5% stearylamine displayed high efficiency of oligonucleotide association as well as considerable stability in the presence of serum, supporting the interest in this new formulation for oligonucleotide delivery[9]. A novel cationic lipid emulsion with promising features for the delivery of plasmid DNA has also been described by Yi et al.[10]. Their emulsion system contained soybean oil as a core oil, DOTAP as a cationic emulsifier and DOPE as a co-emulsifier. $PEG_{2000}PE$ was also added to the oil phase in order to increase the physical stability of the formulation. Results from investigations performed to determine the characteristics of the formulation indicated that this emulsion formed a relatively small and physically stable DNA/emulsion complex displaying highly efficient DNA transfection properties in the presence of up to 90% serum[10]. Recently, Kim and co-workers have also carried out an emulsion-mediated transfection study. They prepared cationic emulsion formulations with various lipids and co-lipids, and compared the transfection activities of emulsions and liposomes made with identical lipid composition omitting the oil component, squalene. It was observed that the transfection activity of the DOTAP/DOPE/Tween 80 emulsion was far greater than that of the counterpart liposome in the presence of serum. Based on the results obtained in this study, the cationic emulsion was suggested to be a more potent gene carrier for in vitro and in vivo applications[14].

In this paper, we report the design and in vitro evaluation results of a cationic emulsion formulation as an alternative pDNA carrier system. Our soybean oil-water emulsion was developed using the combination of 2 emulsifying agents, cetyltrimethylammonium bromide (CTAB) and Pluronic F68. The cationic

surfactant, CTAB, with an HLB value of 10, forms relatively stable emulsions of mineral oil in water, as described previously[15]. The presence of CTAB with a quarternary amine group causes the formation of positively charged droplets that promote strong electrostatic interactions between emulsion and anionic DNA phosphates. Pluronic F68, a nonionic block copolymer of polyoxyethylene-polyoxypropylene, was included in our emulsion formulations since it has been shown that F68 contributes to the stability of emulsion basically through a steric repulsion mechanism[16]. Additionally, the inclusion of Pluronic F68 also significantly affects the serum sensitivity of plasmid DNA/emulsion complex in accordance with a previous report indicating that emulsion containing F68 had a strong resistance to serum[8]. Along with the physical characteristics of the cationic B13d emulsion, we present its ability to associate with pDNA, the protection of complexed pDNA from DNase I digestion and resistance of the complexes to human serum.

Materials and Methods

Materials

Cationic surfactant, cetyltrimethylammonium bromide (CTAB) and DNase I endonuclease were purchased from the Sigma Chemical Company (USA). Nonionic co-surfactant, Pluronic F-68, was a gift from ICI (Belgium). Soybean oil and D,L-α-tocopherol acetate (antioxidant) were obtained from MARSA (Turkey) and Merck (Germany), respectively. The plasmid pGL3, restriction enzyme *Bgl*II, and Wizard® Maxipreps Purification System were supplied by Promega (USA). Agarose and ethidium bromide were obtained from Sigma. The fluorescent dye SYBR Green I was purchased from BMA (USA). Human serum was supplied by Hacettepe University Blood Bank (Turkey). All other chemicals used were reagent grade.

Plasmid DNA purification

The pGL3 plasmid DNA encoding the firefly luciferase reporter gene was amplified in *Escherichia coli* JM109 strain and isolated using the Wizard Maxipreps Purification System according to the manufacturer's instruction. The concentration and purity of plasmid DNA were determined by UV absorbance at 260 nm and 280 nm. The purity of pDNA was also validated by gel electrophoresis after *Bgl*II treatment and one band corresponding to 5.2 kb was observed. pDNA was stored at –20 °C until use.

Interaction of pDNA with cationic surfactant CTAB

A pre-investigation on the amount of the cationic surfactant required to form a stable complex with pDNA was carried out. Reaction mixtures (100 μL) were prepared by adding increasing amounts of CTAB dissolved in distilled water onto 1 μg of pDNA and incubated for 1 h at room temperature. A gel retardation assay was then applied for monitoring the pDNA/CTAB complex formation by loading 15 μL of samples onto a 0.8% agarose gel. After 1 h staining in ethidium bromide, gel photographs were taken under UV light.

Preparation of cationic emulsions

To develop cationic emulsion formulations, the system of ternary phase diagrams was applied to determine the amounts of water, oil, surfactant and co-surfactant for each combination on the diagram. Briefly, the coarse emulsions with various amounts of CTAB as a cationic surfactant and Pluronic F68 as a nonionic co-surfactant were prepared by dissolving CTAB and Pluronic F68 in the aqueous phase containing 2.5%

glycerol. The aqueous phase was then added to the oil phase containing 0.05% D,L-α-tocopherol acetate and emulsification was carried out by stirring the 2 solutions at 2000 rpm by a mechanical stirrer (Heidolph RZR-1, Germany) for 45 min at 21 °C. Samples were homogenized using an ultrasonic probe (Labsonic 2000, Germany) at an intensity of 100-150 W for 30 s to obtain fine emulsions. Following the adjustment of pH to 9.0, the samples were sterilized by autoclaving at 121 °C for 15 min. Visual inspection by polarized light microscopy was performed to examine the structures of the formulations. The presence of fine emulsion, lamellar phase or both were noted. The emulsion stabilities were determined based on phase separation as a function of time.

Characterization of the B13d emulsion

Among the formulations, a cationic oil-in-water emulsion referred to as B13d was found to be stable and the most suitable formulation for parenteral application. B13d, which consisted of 5% CTAB, 5% Pluronic F68, 35% soybean oil and 55% water, was characterized further in terms of its particle size, pH and viscosity. The effects of storage time and temperature on these parameters were investigated at 4, 25 and 40 °C over 5 months.

The particle sizes of B13d as well as the pDNA/emulsion complexes were determined by quasielastic light scattering with a Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). Zeta potential measurements were performed using a Malvern Zetasizer 4 (Malvern Instruments Ltd.). The viscosity of B13d was also measured using an Ubbelohde capillary viscosimeter (Technico, UK).

Determination of the complexation of pDNA and B13d emulsion

Gel retardation Assay: A gel retardation assay was applied to determine the complex formation between pDNA and the cationic B13d emulsion. For this purpose, samples containing 1 μg of pDNA dissolved in TE buffer (20 mM Tris-Cl and 1 mM EDTA pH 7.5) were mixed with increasing amounts of B13d in a total reaction volume of 200 μL. Following incubation for 1 h at room temperature, 10 μL aliquots of the complexes were mixed with 10 μL of loading buffer (0.25% bromophenol blue and 30% glycerol), and samples were loaded onto a 0.8% agarose gel. Electrophoresis was carried out with a current of 70 V for about 2 h in TBE running buffer (100 mM Tris-Cl, 100 mM boric acid and 2 mM EDTA pH 7.5). After 1 h of staining with ethidium bromide, gel photographs were taken under UV light.

SYBR Green I Displacement Assay: The binding ability of the B13d emulsion developed to pDNA was examined by the SYBR Green I displacement assay. Samples were prepared by mixing 3 μg of pDNA with 500 μL of a 1:10,000 TE dilution of SYBR Green I stock, and quenching of SYBR Green I fluorescence intensity by the addition of increasing amounts of B13d was monitored using a fluorescence spectrophotometer (Hitachi 650-40, Japan) at 497 nm excitation and 520 nm emission wavelengths. In this experiment, the background for each sample was determined by measuring the fluorescence of the mixtures containing corresponding amounts of B13d added to 500 μL of a 1:10,000 TE dilution of SYBR Green I in the absence of pDNA. The background values were then subtracted from the readings of the samples.

pDNA/B13d emulsion complex sensitivity to DNase I digestion

pDNA/B13d emulsion complexes were incubated with 50 Kunitz Unit (KU) DNase I for 15 min at 37 °C to determine the sensitivity of the complex to DNase I digestion.

The reactions were terminated by adding 100 μL of 0.1 M EDTA (pH 8.0) to the samples, and subsequently phenol-chloroform extraction was performed to recover pDNAs. The samples were then subjected to agarose gel electrophoresis.

Stability of pDNA/B13d emulsion complex in the presence of serum

To investigate the stability of pDNA/B13d emulsion complex in the presence of serum, naked pDNA and pDNA/B13d emulsion complex containing pDNA:CTAB at the ratio of 1:2.74 (μg/nmol) were incubated with human serum (2.5 times the sample volume) for various periods of time at 37 °C. After the reactions were stopped by adding 10% sodium dodecyl sulfate to the samples, pDNA was recovered by phenol-chloroform extraction. The samples were loaded on an agarose gel, and visualized by ethidium bromide staining.

Results

Interaction of pDNA with Cationic Surfactant CTAB

Prior to the preparation of cationic emulsions, we investigated the interaction between polyanionic pDNA and the cationic surfactant as described in the Materials and Methods section. Agarose gel electrophoresis of the pDNA-CTAB mixtures allowed us to determine the amount of CTAB required to form a complex with 1 μg of pDNA (Figure 1). Mixtures containing pDNA:CTAB (μg/nmol) at ratios of 1:0.0014, 1:0.0027, 1:0.0082, 1:0.0219, 1:0.192, 1:0.205, 1:0.219 (Lanes 4-10) revealed 2 bands corresponding to the super-coiled and circular forms of pDNA, indicating that the pDNA was free to migrate in the gel.

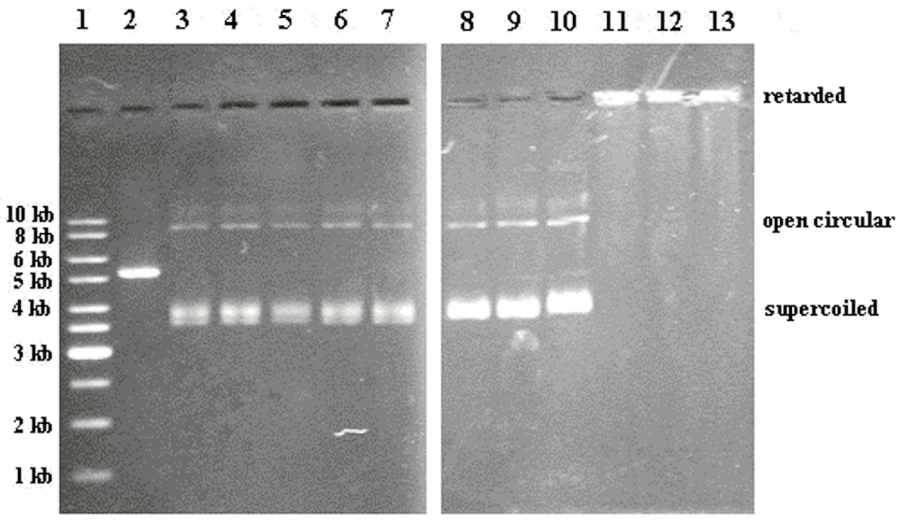

Figure 1. Interaction of pDNA with CTAB. Increasing amounts of CTAB dissolved in water were added to a constant amount of 1 μg of pDNA. The unbound free (open circular and super-coiled) or bound retarded bands of pDNA were visualized under UV light. Lane 1, DNA marker; Lane 2, pDNA digested with *Bgl*II; Lanes 3-13, pDNA:CTAB (μg/nmol) mixtures prepared at ratios of 1:0, 1:0.0014, 1:0.0027, 1:0.0082, 1:0.0219, 1:0.192, 1:0.205, 1:0.219, 1:0.233, 1:0.247 and 1:0.274, respectively.

The retarded pDNA bands in mixtures containing the pDNA:CTAB at ratios of 1:0.233, 1:0.247 and 1:0.274 (Lanes 11-13) were observed as an indication of complex formation. In addition, the complete binding of pDNA with CTAB was confirmed by the absence of free DNA bands in these samples.

Preparation of cationic emulsions

The system of ternary phase diagrams was used to determine the amounts of water, oil, surfactant and co-surfactant for each emulsion formulation in the diagram. Coarse emulsions with various amounts of CTAB as a cationic surfactant and Pluronic F-68 as a nonionic co-surfactant were prepared as described in the Materials and Methods section. Using polarized light microscopy, the structures of the formulations were investigated to determine whether they show the characteristics of fine emulsion, lamellar phase or both. The ternary phase diagram boundaries of the emulsion formulations are indicated in Figure 2.

Among all the formulations prepared, an O/W emulsion containing liquid crystal phase (referred to as B13d) was found to be suitable for our goal and was selected for future preparation of pDNA/emulsion complexes.

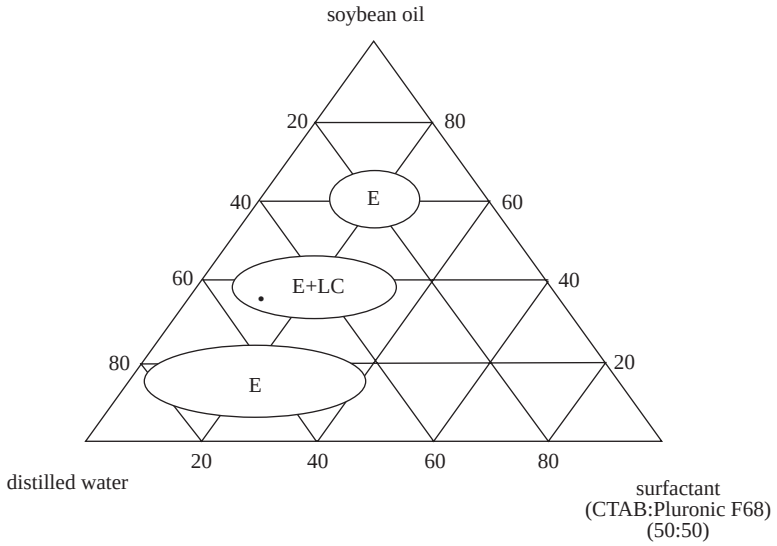

Figure 2. Ternary phase diagram of water/soybean oil/surfactant (CTAB:Pluronic F-68) system. Open-circled regions indicate the boundaries of formulations showing the characteristics of emulsion (E), liquid crystal (LC) or both (E+LC). The filled-circle represents B13d formulation.

Characteristics of the B13d emulsion and pDNA/B13d emulsion complex

Since the visual assessment indicated no phase separation, other physical characteristics of B13d were investigated. Following the determination of the particle size, viscosity and pH of the freshly prepared B13d, the same measurements were also conducted on emulsion samples stored at 4, 25 and 40 °C for a 5-month period (Table). The particle size of the freshly prepared B13d was 350 nm. The effects of storage time and temperature on the particle size of B13d emulsions were observed to be highly divergent. The particle size of the emulsions stored at 4 °C increased gradually with time and reached a maximum of 2.18 μm at the end of the fifth month. At 40 °C, emulsions showed a slight droplet size decrease in the first

month but they then deteriorated, reaching a droplet size of above 5 μm in the second month. On the other hand, although there was a slight decrease in particle size, the emulsions stored at 25 °C were relatively stable over the 2-month period. The longer storage at this temperature resulted in enlargement of droplet size, reaching 2.85 μm at the last measurement. The particle size changes observed at 4 and 25 °C were acceptable according to the desired limits (between 0.5 and 3 μm).

Table. Effect of storage temperature on the mean droplet size, pH and viscosity of B13d with time.

	Mean droplet size (μm)*			pH*			Viscosity (cps)*		
	Temperature (°C)								
	4	25	40	4	25	40	4	25	40
Storage time (months)									
0	0.35 ± 0.0	0.35 ± 0.0	0.35 ± 0.0	7.30 ± 0.0	7.30 ± 0.0	7.30 ± 0.0	64.5 ± 0.0	64.5 ± 0.0	64.5 ± 0.0
1	1.74 ± 0.1	0.22 ± 0.0	0.24 ± 0.0	7.24 ± 0.0	6.78 ± 0.1	6.58 ± 0.0	37.1 ± 0.1	40.2 ± 0.5	42.7 ± 0.4
2	2.02 ± 0.2	0.19 ± 0.0	5.64 ± 0.4	7.37 ± 0.0	7.04 ± 0.0	6.75 ± 0.0	62.2 ± 0.3	39.3 ± 0.3	41.4 ± 0.3
5	2.18 ± 0.1	2.85 ± 0.1	4.89 ± 0.4	7.13 ± 0.1	7.11 ± 0.0	6.39 ± 0.1	61.8 ± 0.2	44.8 ± 0.3	44.0 ± 0.5

*n = 3
Values are mean ± s.d.

It was observed that the pH of emulsions was affected by the storage temperature with time. Especially at 40 °C, a significant decrease in the pH value of the emulsions was observed in the first month. The pH decrease at this temperature is probably due to the formation of fatty acids as a result of the hydrolysis of triglyceride and phospholipid moieties[17]. Nevertheless, the pH change in emulsions was minimal even after 5 months of storage at 4 and 25 °C. The desired pH limits, 7.00-7.50, for i.v. emulsions can be maintained.

In general, the viscosity of emulsion samples stored at 4, 25 and 40 °C decreased with time. The level of viscosity change was, however, more drastic in emulsions stored at 25 and 40 °C. These changes did not affect the flow of the emulsions through the injector needle for administration.

The effect of pDNA association on the particle size of B13d was also investigated after adding pDNA to the freshly prepared emulsion. The particle size of the freshly prepared B13d complexed with pDNA at the pDNA:CTAB ratio of 1:2.74 (μg/nmol) (360 nm) was found to be very similar to that of empty B13d (350 nm).

Zeta potential measurements revealed that pDNA association did not cause a significant change since the value of +43.7 mV was observed with a pDNA-loaded emulsion while the zeta potential value of the empty emulsion was +45.3 mV.

pDNA and B13d emulsion complexation

Gel Retardation Assay: To demonstrate complex formation between pDNA and our cationic B13d emulsion, a gel retardation assay was used. We prepared pDNA and emulsion mixtures by considering the CTAB concentration of B13d. Constant amounts of 1 μg of pDNA and increasing amounts of freshly prepared B13d with the starting CTAB concentration of 0.233 nmol were mixed since the binding of pDNA to CTAB alone was initially determined to occur at a pDNA:CTAB ratio of 1:0.233 (μg/nmol). As shown in Figure 3, unbound free pDNA bands were clearly visible at complex ratios (μg/nmol) of 1:0.233, 1:0.274, 1:0.356, 1:0.411, 1:0.548, 1:1.781, 1:1.918, 1:2.055, 1:2.192 and 1:2.329 (Lanes 2-11). On the other hand, pale fluorescent bands were observed in the wells of the first 6 samples (Lanes 2-7), indicating that some but

not all pDNA formed complex with B13d. In emulsions with the CTAB concentrations of 1:1.918, 1:2.055, 1:2.192 and 1:2.329 (Lanes 4-7), the visibility of the free DNA bands decreased and retarded bands began to disappear, suggesting a higher level of complex formation. Eventually, a complete complexation between pDNA and B13d was achieved at a pDNA:CTAB ratio of 1:2.74 (Lane 12). Since all of the pDNA (1 μg) participated in the complex formation, neither free nor retarded DNA bands were visible in this sample. While the complexes were detectable by eye at the application site in samples with high complex ratios, the EtBr fluorescence was not detectable under UV light (Lanes 8-12). This was unsurprising because the same observation was previously made in a lipid emulsion/DNA complex formation study conducted by Yi et al.[10]. The most likely explanation for this observation is that the cationic emulsion can also condense the pDNA, preventing EtBr from intercalating within the bases of DNA. Additionally, we noted that the open-circular forms of the pDNA participated in the complex formation earlier than the super-coiled forms.

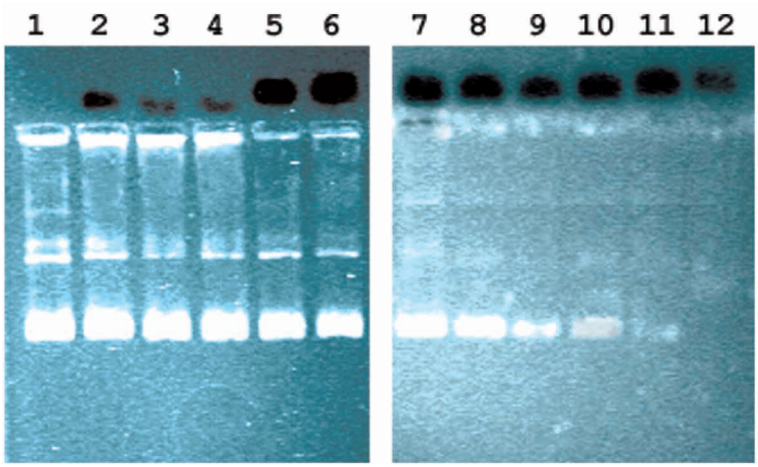

Figure 3. Gel retardation assay of pDNA/B13d emulsion complexes. pDNA (1 μg) was complexed with increasing amounts of cationic B13d emulsion. The pDNA:CTAB ratios in complexes are (μg/nmol): Lane 1, 1:0; Lane 2, 1:0.233; Lane 3, 1:0.274; Lane 4, 1:0.356; Lane 5, 1:0.411; Lane 6, 1:0.548; Lane 7, 1:1.781; Lane 8, 1:1.918; Lane 9, 1:2.055; Lane 10, 1:2.192; Lane 11, 1:2.329; Lane 12, 1:2.740.

SYBR Green I Displacement Assay: The pDNA binding ability of cationic B13d emulsion was also examined using an SYBR Green I displacement assay. While the fluorescence signal of this dye is enhanced when it is intercalated into DNA, its displacement from the intercalation site results in loss of the signal[18]. Thus, quenching of SYBR Green I fluorescence intensity by the addition of increasing amounts of B13d to the pDNA/SYBR Green I complex was monitored using a spectrofluorometer. As shown in Figure 4, the fluorescence intensity of the pDNA/SYBR Green I complex started to decline with the addition of B13d samples containing 0.08, 0.23, 0.55, 0.82, 1.37, 1.92, 2.19 and 2.44 nmol of CTAB. Finally, the CTAB concentration of B13d reached 2.74 nmol, and the intensity fell to the background value, suggesting that all pDNA dissociated from SYBR Green I and formed complex with the cationic B13d emulsion. Based on this assay, the pDNA condensing ability of B13d at the pDNA:CTAB ratio of 1:2.74 was estimated to be around 95%. The SYBR Green I displacement assay thus confirmed the data obtained in the gel retardation assay.

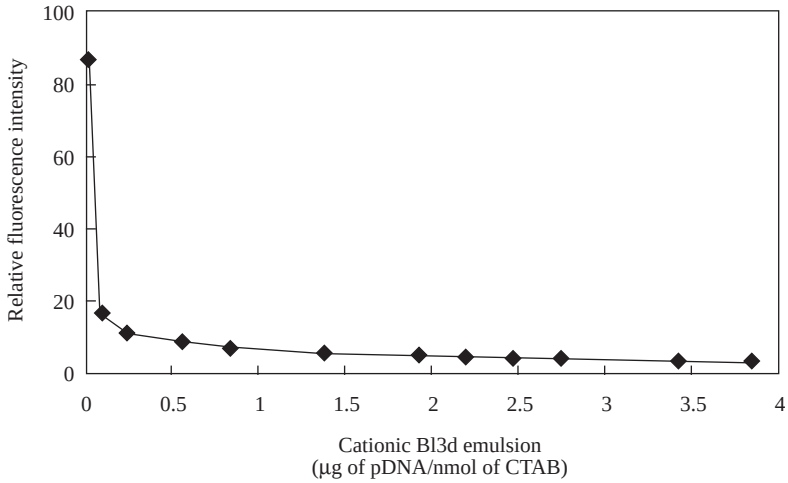

Figure 4. SYBR Green I displacement assay. The fluorescence quenching of SYBR Green I by the addition of increasing amounts of B13d onto the pDNA-SYBR Green I complex was monitored (SYBR Green I excited at 497 nm with emission at 520 nm).

pDNA/B13d emulsion complex sensitivity to DNase I digestion

The sensitivity of pDNA in the complex to DNase I digestion was used as an assay to determine the stability of the pDNA/B13d emulsion complexes. The amount of DNase I and incubation time necessary for degrading 1 μg of naked pDNA were determined as 50 KU and 15 min, respectively (data not shown). The pDNA/B13d emulsion complexes at 2 different mixing ratios were prepared and subjected to DNase I digestion, and undigested pDNA was recovered and visualized as described in the Materials and Methods section. As shown in Figure 5, naked pDNA was completely degraded under the experimental conditions (Lane 2). Unbound free pDNA bands were observed at the complex ratio of 1:0.233 (μg/nmol) (Lane 3), confirming the data obtained by the gel retardation assay. The pDNA in this complex was surprisingly protected from DNase I digestion (Lane 4). When the complex ratio was 1:2.74 (μg/nmol), neither free nor retarded DNA bands were visible (Lane 5), indicating full complex formation as previously observed in the gel retardation assay. After exposure of this complex to DNase I digestion, the pDNA was also found to be highly intact (Lane 6), suggesting that the examined pDNA/B13d emulsion complexes were likely to survive in the presence of serum nucleases as well.

Serum resistance of pDNA/B13d emulsion complex

The nuclease resistance of complexed pDNA was also examined in the presence of serum. For this experiment, naked pDNA and pDNA complexed with B13d at the pDNA:CTAB ratio of 1:2.74 (μg/nmol) were incubated for various time periods in fresh human serum (2.5 times the sample volume). pDNA was extracted from the sample solutions by the phenol/chloroform method and then subjected to agarose gel electrophoresis (Figure 6). There was a substantial decrease in the super-coiled form of naked pDNA within a couple of hours of incubation in the presence of serum, and after 10 h the super-coiled form completely disappeared (Figure 6a). On the other hand, the observation of the super-coiled form of pDNA even after 24 h incubation was evidence of improved nuclease resistance of pDNA by complexation with B13d (Figure 6b).

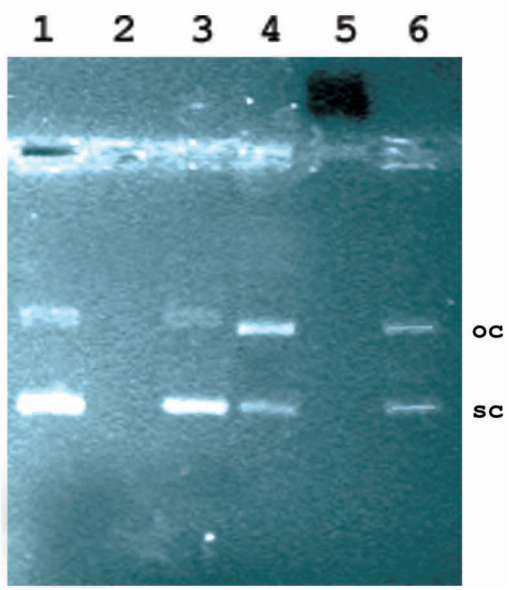

Figure 5. Stability of pDNA/B13d emulsion complexes against DNase I digestion. Lane 1, naked pDNA; Lane 2, naked pDNA incubated with DNase I; Lane 3, pDNA/emulsion complex at the pDNA:CTAB ratio of 1:0.233 (μg/nmol); Lane 4, pDNA/emulsion complex (1:0.233) incubated with DNase I; Lane 5, pDNA/emulsion complex at the pDNA:CTAB ratio of (1:2.74); Lane 6, pDNA/emulsion complex (1:2.74) incubated with DNase I. (oc, open circular; sc, super-coiled pDNAs).

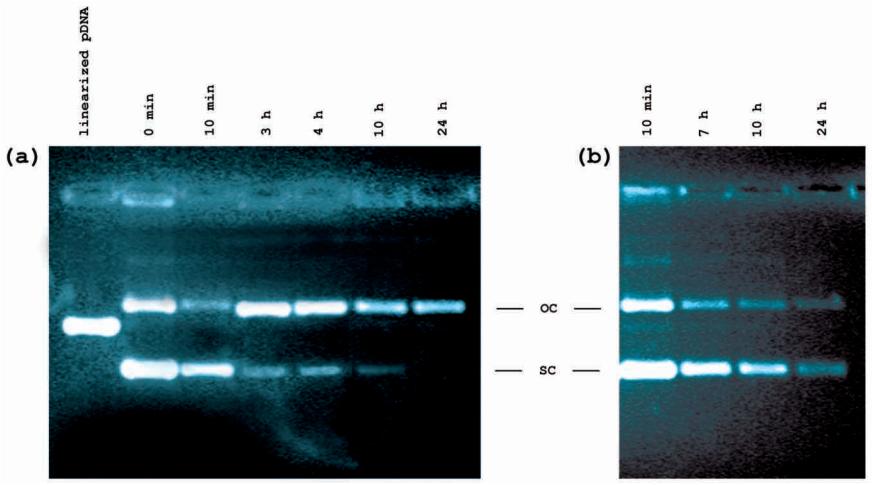

Figure 6. Serum resistance of pDNA/B13d emulsion complex. **a)** Naked pDNA incubated with human serum. **b)** pDNA complexed with B13d at the pDNA:CTAB ratio of 1:2.74 (μg/nmol) incubated with human serum. (oc, open circular pDNA; sc, super-coiled pDNA).

Discussion

Since the description of positively charged submicron emulsions as useful drug carriers for the delivery of various therapeutic agents[19], cationic emulsions have also been investigated as a vehicle for gene delivery. Knowing that the formulation of stable emulsions requires the use of an emulsifier blend[20], we used a

combination of 2 emulsifying agents, Pluronic F68 and CTAB, to formulate a cationic emulsion for the purpose of gene delivery.

The nonionic co-emulsifier used as a stabilizing agent in our formulations was Pluronic F68. Pluronics, also named poloxamers, are regarded as nontoxic and nonirritant materials and are used in a variety of oral, parenteral and topical pharmaceutical formulations [21].

The choice of CTAB was based on its ability to form relatively stable emulsions and to provide the positive charge required for DNA binding. In cosmetics and pharmaceutical formulations, such as in eye-drops, it is used as an antimicrobial preservative[22]. CTAB has also been previously used in various gene and immunogene delivery systems as an ion-pairing agent. The earliest report on the use of CTAB in gene delivery systems came from the laboratory of Huang, who investigated the effect of quarternary ammonium detergent (dodecyl-, tetradecyl- or cetyl-trimethylammonium bromide) in liposome-mediated DNA transfection of mouse L-cells. Analysis of the 3 detergents in combination with the lipid showed that cetyltrimethylammonium bromide was least toxic to the cells, and CTAB, at a minimal concentration of 20 mol% in DOPE, allowed for stable liposome preparations and efficient transfection[23]. Later on, CTAB was used by Fattal et al. for adsorption of oligonucleotides onto polyalkylcyanoacrylate nanoparticles. As a result of both in vitro cell culture experiments and in vivo studies with mice, this system has been proved to be efficient for the specific delivery of antisense oligonucleotides[24]. Berton and co-workers also used CTAB for the production of oligonucleotide hydrophobic complexes encapsulated into poly (D, L-lactic acid) nanoparticles for the delivery of antisense oligonucleotides in 2 separate studies with different purposes. The result from their first study involving the evaluation of the uptake of oligonucleotide-loaded nanoparticles in DV145 prostatic cancer cells indicated that the use of NP improved the compartmentalization of oligonucleotides[25]. In the second study, an NP system was used to inhibit HIV-1 reverse transcriptase activity in HIV-1-infected lymphatic cells. The experimental results of this study suggested that poly (D, L-lactic acid) nanoparticles could be used for improving the cellular delivery of oligonucleotides in HIV natural target cells[26]. Eventually, as a delivery system for DNA vaccines, Singh et al. developed microparticles with a cationic surface through the inclusion of CTAB in the preparation process. With the use of these microparticles presenting DNA on the surface, a significant improvement in immunogenicity was achieved for both antibody and cytotoxic T lymphocyte induction in mice. The optimal enhancement of immune responses was observed with PLG/CTAB microparticles at the 10-μg DNA dose, and preliminary studies in guinea pigs indicated no acute toxicity problems when microparticles were applied at a dose of 1 mg of DNA per animal[27].

Our analysis of the physical characteristics of cationic B13d emulsion before and after complexation with pDNA indicated that the average particle size of freshly made B13d was 350 nm, and the incorporation of pDNA caused a negligible change in droplet size since the pDNA/B13d emulsion complexes were only 360 nm. Particle size affects transfection efficiency of DNA carriers, and particles 300-700 nm in diameter are reported to be more effective than small particles 50-100 nm in size[28]. As a result, the proposed in vitro cell culture experiments will demonstrate whether pDNA/B13d emulsion complexes with a droplet size of 360 nm possess good transfection activity. It is, however, important to note that freshly prepared B13d emulsion samples were used in all analysis performed in our study, and thus it is necessary to prepare the complex freshly and use it within a short period of time. On the other hand, the decrease in pH from 7.30 to 6.58 observed in emulsion samples stored for a month at 40 °C raises the question of whether such a pH change occurs when freshly made complexes are exposed to 37 °C for gene transfer studies. Knowing that gene expression is generally assayed 24-48 h after transfection in many in vitro and in vivo transfection

experiments[4,7,10,14,29,30], the pH of freshly prepared complexes is unlikely to decrease drastically during a relatively short incubation period at 37 °C.

The surface charge of the pDNA/emulsion complexes is also expected to influence their entry into cells. In vitro studies revealed that effective transfection of most cell types requires particles formulated to carry a net positive charge[28,31]. Generally, positively charged liposome-DNA complexes are known to show good transfection activity based on the fact that such complexes bind to the cell surface due to an electrostatic interaction between the positive charges of the cationic liposomes and the negative charges on the cell surface[32−34]. Based on our measurements the zeta potential value of empty B13d was +45.3 mV, indicating that the interfacial film consisting of CTAB and Pluronic F68 formed at the o/w interface resulted in a positive surface charge. As we expected, such a positive charge on the droplets allowed efficient adsorption of pDNA as demonstrated by gel retardation and SYBR Green I displacement assays. Additionally, incorporation of pDNA did not alter the surface characteristic of our emulsion because the zeta potential value of +43.7 mV was noted after the addition of 1 μg of pDNA. The zeta potential of B13d was close to the values of emulsions previously developed by Yi et al. and Klang et al.; the zeta potentials of their emulsions were +50 mV and +41.06 mV, respectively[10,35].

Complex formation between pDNA and the cationic B13d emulsion occurred at the pDNA:CTAB ratio of 1:2.74, which was approximately 10-fold higher than the ratio of the pDNA-CTAB complex in water (1:0.233 μg/nmol). Two possible explanations for such a discrepancy are: *i*) the microenvironment in the aqueous CTAB solution is most likely different than that in the emulsion. In aqueous media, there may be hydrophobic interactions between the pDNA and the lipophilic tail of CTAB as well as ionic interactions with the positively charged head group, resulting in strong interactions even at lower concentrations of CTAB. On the other hand, pDNA is exposed to a totally different environment in B13d containing a 1:1 (w/w) mixture of CTAB and Pluronic F-68. The presence of a nonionic surfactant might modify the surface hydrophobicity of the oil droplets, and thus a higher concentration of CTAB is required to form a stable complex with the same amount of pDNA. *ii*) The nature of the oil phase could also have some influence on the characteristics of the emulsion as it has been previously reported that oil phase can also interact with the emulsifier film[36].

Since serum nucleases degrade naked DNA, a DNA carrier system must also protect DNA from nuclease attack. To test the sensitivity of pDNA/B13d emulsion complexes to nucleases, complexes were exposed to DNase I. Results of this analysis indicated that while pDNA associated with B13d remained highly intact in the presence of DNase I, naked pDNA was completely digested with an equal amount of DNase I.

B13d formulation seems to offer serum resistance that is critical for systemic application. This was supported by the fact that most pDNA in the complex was protected from DNase I digestion. It is known that the transfection by cationic liposomes is sensitive to the presence of serum and this is considered one of the limitations to their applications. The serum stability of the pDNA/B13d emulsion complexes observed under our experimental conditions indicates the potential of this system as a gene carrier. On the other hand, the conversion of pDNA from super-coiled to circular form observed during incubation in the presence of serum raised the question of how changes in plasmid structure might affect the gene transfer efficiency. Based on the results previously reported, such conversion in fact had only a minor effect on the gene transfer efficiency of peptide-condensed pDNA formulations[37]. Additionally, Bergan et al. recently reported that there was no difference in the ability of super-coiled and relaxed pDNA to transfer a variety of cells when they used an assay of cationic lipid transfection reagents[38].

In conclusion, the physical characteristics, pDNA condensing ability and serum stability of the developed cationic formulation are important features suggesting that B13d might be a suitable carier for gene and/or immunogene delivery. Further investigations on the transfection efficiency of B13d will help to validate the potential of this formulation as a new vehicle for gene transfer into various cells.

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