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Research paper

# Topical non-invasive gene delivery using gemini nanoparticles in interferon-γ-deficient mice

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**Abstract**

Cutaneous gene therapy, although a promising approach for many dermatologic diseases, has not progressed to the stage of clinical trials, mainly due to the lack of an effective gene delivery system. The main objective of this study was to construct and evaluate gemini nanoparticles as a topical formulation for the interferon gamma (IFN-γ) gene in an IFN-γ-deficient mouse model.

Nanoparticles based on the gemini surfactant 16-3-16 (NP16-DNA) and another cationic lipid cholesteryl 3β-(-*N*-[dimethylaminoethyl] carbamate) [Dc-chol] (NPDc-DNA) were prepared and characterized. Zetasizer measurement indicated a bimodal distribution of 146 and 468 nm average particle sizes for the NP16-DNA (ζ-potential +51 mV) nanoparticles and monomodal distribution of 625 nm (ζ-potential +44 mV) for the NPDc-DNA. Circular dichroism studies showed that the gemini surfactant compacted the plasmid more efficiently compared to the Dc-chol. Small-angle X-ray scattering measurements revealed structural polymorphism in the NP16-DNA nanoparticles, with lamellar and *Fd3m* cubic phases present, while for the NPDc-DNA two lamellar phases could be distinguished. *In vivo*, both topically applied nanoparticles induced higher gene expression compared to untreated control and naked DNA (means of 0.480 and 0.398 ng/cm$^2$ vs 0.067 and 0.167 ng/cm$^2$). However, treatment with NPDc-DNA caused skin irritation, and skin damage, whereas NP16-DNA showed no skin toxicity.

In this study, we demonstrated that topical cutaneous gene delivery using gemini surfactant-based nanoparticles in IFN-γ-deficient mice was safe and may provide increased gene expression in the skin due to structural complexity of NP16 nanoparticles (lamellar–cubic phases).


*Keywords:* Interferon-γ; In vivo; Gene delivery; Non-invasive cutaneous therapy; Gemini nanoparticles; Interferon-γ-deficient mice; Polymorphic phase; Cubic phase; Circular dichroism; Small-angle X-ray scattering

## 1. Introduction

The skin is the most accessible of somatic tissues for drug delivery. The molecular characterization of several cutaneous disorders reveals the potential of using gene therapy in these diseases [1–4]. However, the cutaneous delivery of genetic material is greatly hindered by the large molecular weight, low diffusion constant and highly hydrophilic nature of DNA. Poor protein expression in the skin cells arises from membrane barriers and enzymatic processes. In addition to the limited penetration through the stratum corneum, gene expression is hindered by slow uptake of the plasmid DNA (pDNA) by the target cells, lack of release from endosomes, instability in cytoplasm and lack of nuclear uptake [5]. However, keratinocytes have been transfected efficiently *ex vivo* and *in vivo* [6] and sufficient amount of protein can be expressed (“bioreactor” concept), which would make topical administration an attractive approach for gene therapy [7].

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Currently, over 600 ongoing clinical trials in the US (http://www.clinicaltrials.gov/) are focusing on both gene therapy and genetic immunization. Although most are oriented towards cancer therapy, some trials are exploring treatment options for other genetic or acquired diseases. Presently, no ongoing clinical trials involve gene delivery to intact skin for treatment of skin disorders. However, in the past decade more emphasis was placed on developing better gene delivery systems and extension of gene therapy to genodermatoses. Although biologically active proteins in lipid-based systems could be delivered through skin [8,9], they are relatively unstable molecules and require intact tertiary and quaternary structure for their activity, whereas pDNA is more stable and a loss of potency is usually due to chemical degradation [10]. An efficient DNA delivery system has to fulfill several criteria: (i) have an ability to bind DNA and create complexes of a certain size and morphology, (ii) neutralize surface charges to facilitate cellular uptake, (iii) induce changes in the DNA structure favorable for efficient delivery, (iv) protect the genetic material against intracellular degradation, and (v) have an ability to undergo polymorphic phase changes [11]. Cationic gemini surfactants are a novel category of delivery agents with potential use in gene therapy. These surfactants are built from two ionic head groups which are attached to their hydrocarbon tails and also connected to each other with a spacer or linker [12]. The gemini surfactants are capable of compacting DNA and have several advantages compared to classic monovalent counterparts: lower cellular toxicity [13], lower critical micelle concentration (generally one or two orders of magnitude), higher efficiency in reducing surface tension, greater tendency to self-assemble, and greater structural variety [14]. DNA compaction is one of the most important requirements for efficient cellular delivery [11]. In addition, for cutaneous gene therapy the DNA has to partition into the viable layers of the skin, overcoming the barrier function of the stratum corneum.

Several studies have demonstrated the efficiency of lipid-mediated delivery of DNA *in vitro* [15–17], including delivery into keratinocytes [18,19] and fibroblasts [20,21]. It also has been shown that cutaneous gene delivery is achievable. Naked DNA was delivered into the skin after the stratum corneum had been altered by chemical [22] or physical [23] methods. Ethanol-in-fluorocarbon microemulsion [24] and a biphasic formulation [25] were used for dermal delivery of genes *in vivo* through intact skin. To date, the efficiency of non-invasive cutaneous gene delivery is still low. In an earlier study [26], we have evaluated the *in vitro* transfection efficiency of a series of gemini surfactant-based delivery systems and demonstrated the feasibility of cutaneous gene delivery through intact skin in normal CD1 mice. In this paper, we evaluate the physicochemical properties of one selected cationic gemini surfactant (16-3-16)-based DNA nanoparticle, and its use in non-invasive topical IFN-γ gene delivery in interferon-γ-deficient mice.

## 2. Materials and methods

### 2.1. Plasmids

The pGTmCMV vector with the murine cytomegalovirus (CMV) promoter designed for gene therapy was used, and the genes of IFN-γ and GFP were inserted in a bicistronic system [26]. Reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA), Qiagen (Mississauga, ON) and restriction enzymes from Amersham Pharmacia Biotech (Baie d'Urfe, QB).

### 2.2. Topical nanoparticle (NP) delivery systems

A topical nanoparticle delivery system (NP16) was prepared with the gemini cationic surfactant *N*,*N*′-bis(dimethylhexadecyl)-1,3-propanediammonium dibromide (16-3-16). The nanoparticles were prepared using 1,2 dioleyl-sn-glycero-phosphatidylethanolamine (DOPE), 10 mg/mL, 1,2 dipalmitoyl-sn-glycero-phosphatidylcholine (DPPC) (Sigma) 10 mg/mL, the gemini surfactant, 10 mg/mL, and diethylene glycol monoethyl ether (Gattefossé, Saint-Priest, France), 25 mg/mL in distilled water. As a control, cholesteryl 3β-(-*N*-[dimethylamino-ethyl] carbamate), 10 mg/mL (Dc-chol, Sigma), replaced the gemini surfactant (NPDc formulation). The DNA concentration in the formulation (NP16-DNA and NPDc-DNA) was 25 μg plasmid/50 μL.

### 2.3. Atomic force microscopy (AFM)

AFM images were obtained with a Pico SPM instrument (Molecular Imaging Inc., Tempe, AZ), with MAC-mode, using MI MAC cantilever Type II ($K = 1.2$–$5.5$ N/m). The topical formulations (10 μl each) were spread on the surface of freshly cleaved mica (Grade V-4, SPI Supplies, West Chester, PA) and incubated for 15 min at room temperature. Excess formulation was removed with lint free absorbent tissue, and then surface dried with $N_2$ gas. A $4 \times 4$ μm area was scanned.

### 2.4. Size and zeta-potential (ζ) measurement

Topical nanoparticles were prepared as described under Section 2. The pGTmCMV.IFN-GFP plasmid was prepared at 500 μg/mL concentration. The size of the particles and ζ-potential were measured with a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Particle sizes and ζ are reported as the mean ± standard deviation.

### 2.5. Circular dichroism (CD)

Aqueous DNA solution and topical nanoparticles were prepared as described under Section 2. All formulations were diluted 10-fold prior to measurement and degassed at 37 °C in a bath sonicator. Spectra were obtained by

using an Applied Photo Physics π* 180 instrument (Leatherhead, UK) with a 4 nm slit, at 25 °C.

### 2.6. Small-angle X-ray scattering (SAXS)

Topical nanoparticles were prepared as described under "Topical formulations". Samples were loaded into 1.5-mm borosilicate capillaries (Charles Supper, Natick, MA). Scattering was measured using the synchrotron light source (Beam X21A, National Synchrotron Light Source, Brookhaven, NY) [27]. Peak fitting of the radially averaged data was carried out using the software *fityk* (http://www.unipress.waw.pl/fityk/).

### 2.7. Topical treatment

The animal experiments were approved by the University of Saskatchewan Committee on Animal Care and Supply Protocol Review Committee. For *in vivo* delivery, two sets of 8–10-week-old female IFN-γ-deficient mice (B6.129S7-IFNg$^{tm1\ Ts}$/J, The Jackson Laboratory, Bar Harbor, ME) were used. All animals (including controls) were anesthetized with isoflurane and close-shaved a day prior to treatment. For the treatments, animals were anesthetized with acepromazine 5 mg/kg and ketamine 100 mg/kg injected intraperitoneally. The shaved area was cleaned with distilled water using sterile gauze and dried. Naked DNA solution or nanoparticle DNA formulations (50 μL containing 25 μg pGTmCMV.IFN-GFP plasmid for each animal) were painted on the shaved area, using a pipette, and covered with parafilm for 2–3 h. The animals were restrained in individual cages for 3 h to prevent removal of the patches and allow the treatment to absorb. Treatments were repeated for four days at 24-h intervals. Each animal received a total dose of 100 μg pGTmCMV.IFN-GFP plasmid. Skin biopsies were collected 24 h after the last treatment. The samples were stored at −80 °C.

### 2.8. ELISA

Protein was extracted and ELISAs were performed as described earlier [26]. Briefly, round-bottomed 96-well plates (Immulon II, Dynatech Laboratories, Chantilly, VA), were coated with rat anti-mouse IFN-γ capture antibody (2 μg/mL) and incubated for 24 h at 4 °C. The wells were blocked with 1% bovine serum albumin (BSA) (New England Biolabs, Mississauga, ON) in PBS at room temperature for 1 h. Protein extracts from skin homogenates

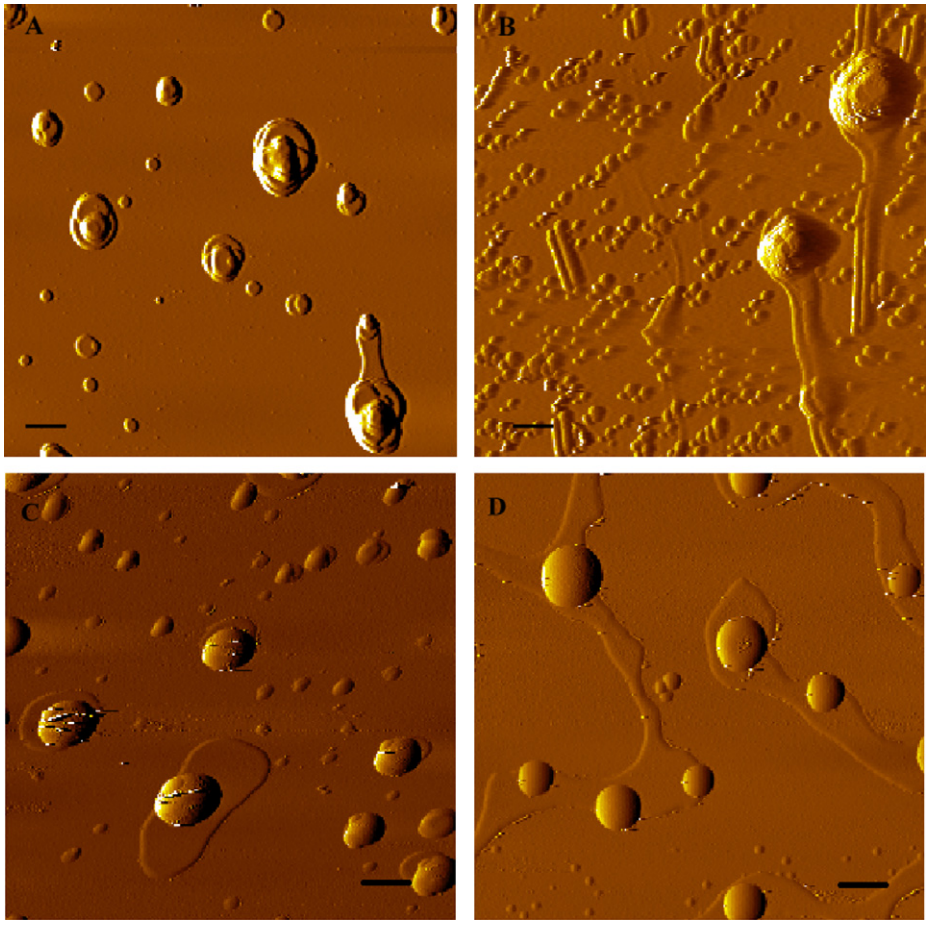



Fig. 1. AFM images of the nanoparticles prepared with the 16-3-16 surfactant (A – blank and B – DNA formulation) and with Dc-Chol (C – blank and D – DNA formulation) used for topical treatment of mice. Bar 400 nm.

were diluted one in four on plates. The plates were incubated overnight at 4 °C. Biotinylated rat anti-mouse IFN-γ (0.5 ng/mL) was added. The plates were incubated for 2 h at room temperature. The streptavidin–alkaline phosphatase conjugate (Invitrogen Life Technologies, Carlsbad, CA) was added in 1:1000 dilution and incubated for 1 h at room temperature. The 4-nitrophenyl phosphate di(tris) salt 1 mg/mL in PNPP buffer (1% diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) (Sigma) was used to develop the assay. Optical density of the samples was measured at 405 nm, using Microplate Reader Benchmark instrument (Bio-Rad, Mississauga, ON), and the concentration of the IFN-γ was calculated from standardized IFN-γ curve. To compare the two experiments, average IFN-γ levels from the untreated animals were subtracted from individual values. Antibodies and recombinant murine IFN-γ were purchased from Pharmingen, Mississauga, ON.

### 2.9. Statistical analysis

Statistical analysis was performed by ANOVA test and the groups were compared using Tukey's multiple comparison test using SPSS 13.1 software.

## 3. Results

### 3.1. Characterization of the cationic gemini surfactant-based nanoparticles (NP)

In this study we used gemini nanoparticles comprised of the 16-3-16 gemini surfactant, as this compound has shown the highest transfection efficiency *in vitro* in our previous work [26]. In addition, we included another formulation based on a known cationic lipid for comparison. DNA formulation prepared with Dc-chol, a cationic cholesterol-derivative, that has been used as an effective and safe cationic lipid for topical gene delivery to nasal epithelia in animal studies [28,29] and cystic fibrosis patients [30], was intended as an *in vivo* positive control.

#### 3.1.1. Size and morphology of the nanoparticles

AFM imaging of the blank NP16 formulation indicated the presence of vesicle-like particles ranging in size from 100 to 500 nm (Fig. 1A). In the DNA nanoparticles (Fig. 1B), the majority of particles were 100–200 nm, indicating a well-compacted DNA-cationic surfactant complex structure. The formation of a fairly homogeneous particle population was observed, as only a few larger, vesicle-like particles were seen in the NP16-DNA formulation. While the blank Dc-chol formulation (Fig. 1C) was similar to that of blank NP16 nanoparticles, the average particle size in the NPDc-DNA formulation (Fig. 1D) was generally higher compared to the NP16-DNA formulation. Light scattering size measurement results of gemini nanoparticles are in agreement with the AFM results, plots of the average particle size distributions are shown as volume distribution (the area of the peak indicating the volume of a certain population of particles) in Fig. 2. The blank NP16 formulation exhibited a particle population having a mean size of 219 ± 49 nm (2–4 measurements of three separate batches) that contribute 55% of the total scattering volume. The blank Dc-chol formulation had a comparable particle size of 211 ± 66 nm (2–4 measurements of three separate batches) that contributed 42% of the total scattering volume. The addition of DNA to NP16 resulted in an essentially bimodal distribution for the main scattering population with average sizes of 148 ± 41 and 468 ± 115 nm (2–3 measurements of four separate batches) contributing 29% and 57% of the scattering intensity, respectively. The NPDc-DNA formulations had a much larger average particle size of 625 ± 99 nm (2–3 measurements of four separate batches) contributing 67% of the scattering intensity. ζ-potential measurements indicated a positive ζ-potential for both

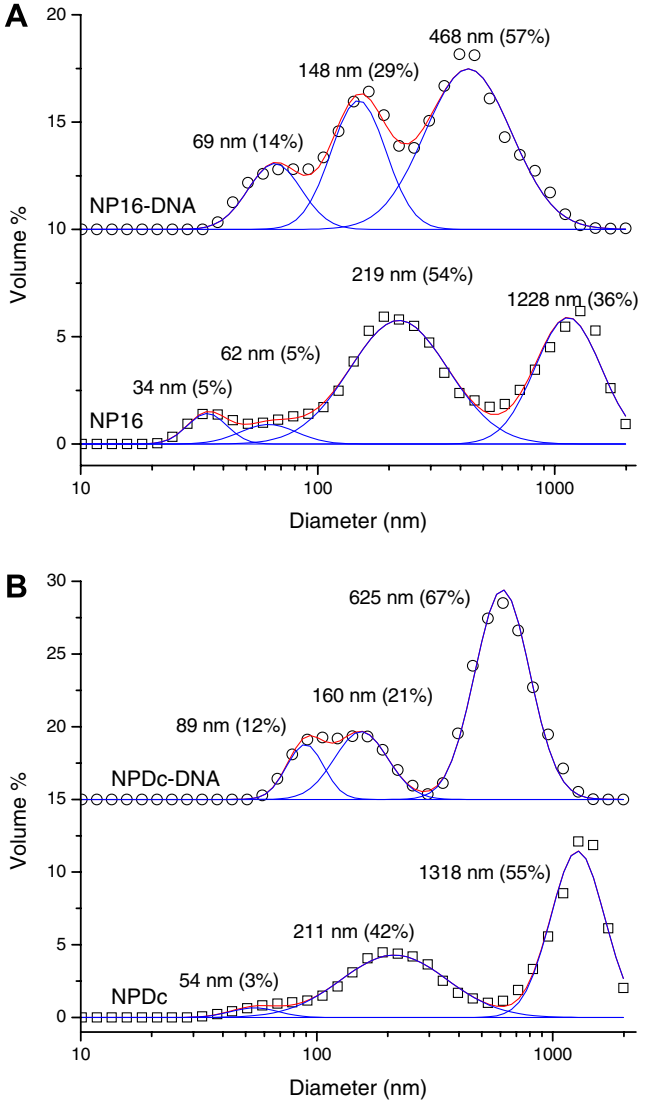



Fig. 2. Average particle size distributions for the (A) NP 16 ($n = 12$), NP16-DNA ($n = 12$), and (B) NPDc ($n = 7$) and NPDc-DNA ($n = 9$) formulations. Peaks were fit with Microcal OriginPro 7.5 peak fitting module.

blank formulations; $64 \pm 5$ and $62 \pm 5$ for the NP16 and NPDc formulations, respectively. As expected, the addition of the negatively charged plasmid DNA reduced the ζ-potential by 20–30%, but the excess of cationic surfactant (negative to positive charge ratio of approximately 1:20) rendered a positive ζ-potential to the gemini and Dc-chol-based DNA nanoparticles of $51 \pm 2$ and $44 \pm 7$ mV, respectively.

### 3.1.2. Interactions between the DNA and nanoparticles

The CD spectrum of the NP16-DNA and NPDc-DNA nanoparticles (Fig. 3) indicated that the cationic agents induced changes in the DNA structure similar to those obtained for the *in vitro* transfection systems [26], namely modifications in the 290 and 260 nm peaks. While Dc-chol caused flattening of the 290-nm peak and a shift of the 260-nm peak into the negative region, the gemini surfactant completely abolished the 290-nm peak, and caused marked depression of the 260-nm peak, increasing significantly the overall negative ellipticity of the DNA. This indicates that the gemini surfactant markedly increased interhelical interactions in the DNA strands [31], giving rise to a more compact structure.

The X-ray scattering profiles for the blank topical formulations (Fig. 4A) were very similar in all cases and consist of a single broad feature, characteristic of interparticle interactions between the nanoparticles in the formulation. No long-range order was evident. The position of the scattering peaks and the corresponding *d*-spacings ($d = 2\pi/q$) are given in Table 1. The addition of plasmid DNA to the topical formulations resulted in significant changes in the scattering profiles; most notably an increase in the order of the bilayers as evidenced by the presence of numerous additional scattering peaks now superimposed on the broad scattering profile for the blank formulation (Fig. 4B). In order to better visualize the scattering peaks induced in the formulations containing plasmid, the profile for the blank formulation was subtracted from the active

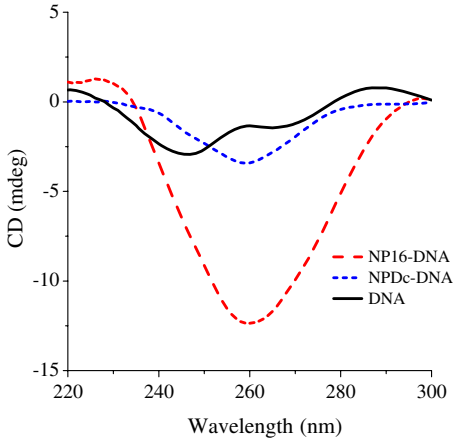



Fig. 3. CD spectra of the naked DNA and DNA-nanoparticles prepared with the gemini surfactant or Dc-chol. Formation of $\Psi^-$ DNA, a highly compacted DNA structure, is crucial for efficient cellular uptake.

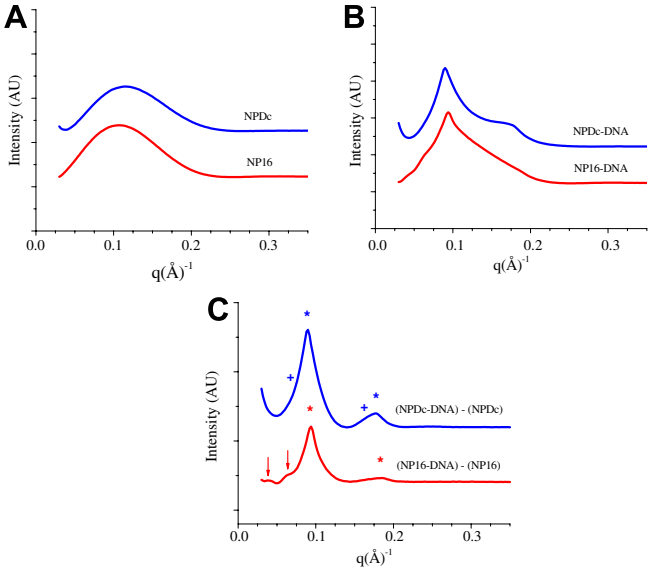



Fig. 4. SAXS profiles of the topical nanoparticles. (A) Blank topical nanoparticles. (B) Topical DNA-nanoparticles. (C) Scattering profiles for the topical formulations containing plasmid, corrected for the scattering arising from the blank formulations. Peaks corresponding to lamellar phases are indicated by * and peaks corresponding to the *Fd3m* cubic phase are indicated by ↓. For the NPDc system, $^+$indicates the presence of a second lamellar phase.

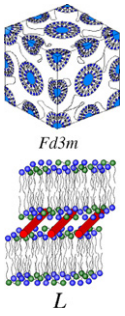
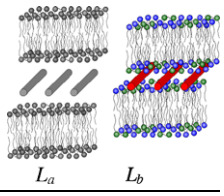
formulations (i.e., containing plasmid) giving the profiles shown in Fig. 4C. The NP16-DNA gemini nanoparticles appeared to consist of two phases; a lamellar phase (indicated by asterisks) and a second phase that appeared as much weaker scattering peaks (indicated by arrows). This second phase is consistent with the *Fd3m* cubic phase, although due to lack of higher order reflections this assignment is qualitative. The cubic lattice constant (*a*) ($a = \frac{2\pi\sqrt{h^2+k^2+l^2}}{q}$, where *h*, *k*, and *l* are the Miller indices) is given in Table 1. In the Dc-chol formulation (NPDc-DNA), a second lamellar phase was present (indicated by the plus sign).

### 3.2. Study on the extent of gene expression in IFN-γ-deficient mice

Following the preliminary studies in CD1 mice [26], where background levels of IFN-γ can contribute to the overestimation of our therapeutic IFN-γ gene, we wanted to evaluate gene delivery in a more suitable model such as the IFN-γ-deficient mouse. The IFN-γ-deficient strain was created by homozygous targeted mutation. These animals are unable to synthesize IFN-γ, appear normal in a clean environment, but cannot mount immune response to external stimuli [32].

Topical treatment with the pGTmCMV.IFN-GFP in gemini nanoparticles or in Dc-chol formulation in the IFN-γ-deficient mice resulted in significantly higher levels of IFN-γ expression compared to the control (0.067 ng/cm$^2$) ($p < 0.01$ and $p < 0.05$, respectively)

Table 1
Scattering peak positions and *d*-spacings for the topical formulations

| Formulation | $q$ (Å$^{-1}$) | Phase | $d$ (Å) | $a$ (Å) | |
|---|---|---|---|---|---|
| NP16 | 0.106 | | 59.3 | | |
| NP16-DNA | 0.040 | *Fd3m* | | 275 |  |
| | 0.064 | *Fd3m* | | | |
| | 0.094 | *L* | 67.0 | | |
| | 0.187 | *L* | | | |
| NPDc | 0.114 | | 55.1 | | |
| NPDc-DNA | 0.080 | $L_a$ | 78.7 | |  |
| | 0.090 | $L_b$ | 69.5 | | |
| | 0.163 | $L_a$ | | | |
| | 0.177 | $L_b$ | | | |

(Fig. 5). The protein expression in animals treated topically with plasmid DNA solution was generally low (mean 0.167 ng/cm$^2$). When comparing the two topical formulations to treatment with naked DNA (in solution), the gemini nanoparticles (NP16-DNA-t) showed a three-fold increase in transgene expression (0.480 ng/cm$^2$ vs. 0.167 ng/cm$^2$; $p = 0.088$). The effect after NPDc-DNA application (0.398 ng/cm$^2$) was not statistically different from the treatment with naked DNA ($p = 0.344$). Skin toxicity observations showed that treatment with NPDc-DNA resulted in skin irritation and redness (6/6) whereas none of the animals of the other topical treatments, DNA solution and gemini nanoparticles had any signs of abrasion or irritation (0/5) (Fig. 6).

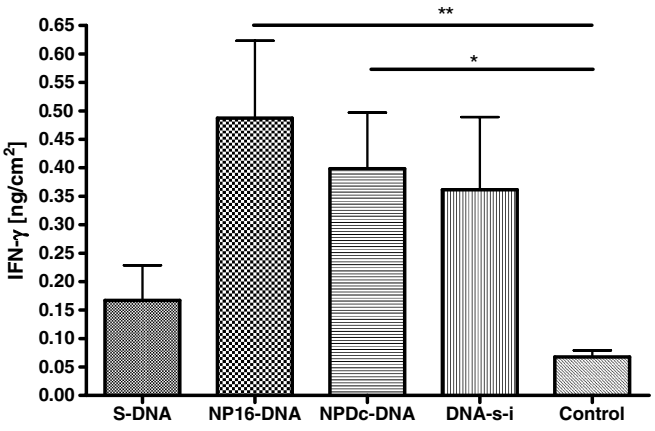



Fig. 5. IFN-γ expression in IFN-γ-deficient mice treated topically with plasmid DNA solution and with DNA-nanoparticles. Bars show the mean ± SEM. ANOVA and Tukey's multiple comparison showed significant differences were observed between the two topical formulations and the control group at the $*p < 0.05$ and $**p < 0.01$ level, respectively; S-DNA vs NP16-DNA $p = 0.088$; S-DNA vs NPDc-DNA $p = 0.344$. S-DNA – topically treated with pGTmCMV.IFN-GFP, 25 μg in 50 μL of aqueous solution, for four days (total dose 100 μg); $n = 9$. NP16-DNA – topically treated with pGTmCMV.IFN-GFP in cationic gemini 16-3-16 nanoparticles, 25 μg in 50 μL, for four days (total dose 100 μg); $n = 9$. NPDc-DNA – topically treated with pGTmCMV.IFN-GFP in Dc-chol nanoparticles, 25 μg in 50 μL, for four days (total dose 100 μg); $n = 9$. S-DNA-i – animals injected with 2.5 μg pGTmCMV.IFN-GFP in aqueous solution at four sites (total dose of 10 μg); $n = 9$. Control – untreated skin samples; $n = 16$.

## 4. Discussion

In this study, topical gemini nanoparticles were characterized and their potential in gene therapy was evaluated. Transgene expression in the skin of the animals treated with NP16-DNA nanoparticles was higher compared to animals treated with naked DNA. The IFN-γ levels measured in the IFN-γ-deficient mice confirmed that the gemini nanoparticles were effective in delivering the plasmid. IFN-γ levels were comparable to levels measured in normal CD1 mice in both DNA solution and gemini nanoparticle formulation [26].

The NP16-DNA formulations prepared with the micelle-forming gemini surfactant showed similar transgene expression, compared to the NPDc nanoparticles prepared with the bilayer stabilizing cholesterol derivative. However, NPDc nanoparticles caused skin irritation that compromised the integrity of the stratum corneum, which probably allowed plasmid DNA to permeate through the damaged skin, and the detected levels of gene expression are a reflection of this.

In the previously used CD1 mouse strain, where skin irritation was not observed, the Dc-chol nanoparticles induced low levels of transgene expression (0.082 ng/cm$^2$) [26]. This may indicate that gemini surfactant-based nanoparticle structure promotes more efficient DNA transport

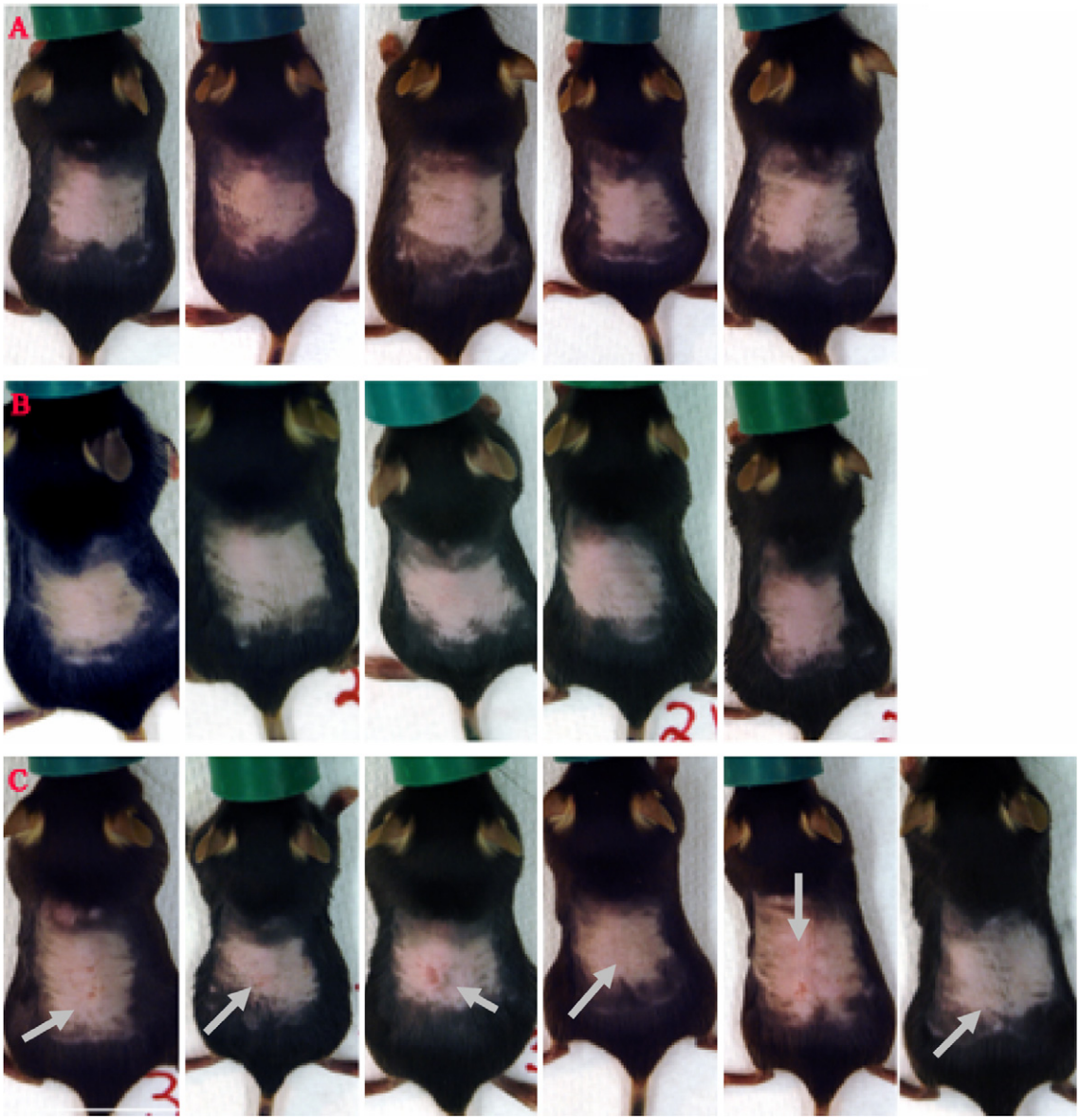



Fig. 6. Photographs taken of the shaved area of skin after a four-day treatment. (A) Mice treated topically with pGTmCMV.IFN-GFP, 25 μg in 50 μL of aqueous solution, for four days (S-DNA); (B) mice treated topically with pGTmCMV.IFN-GFP in cationic gemini 16-3-16 nanoparticles, 25 μg in 50 μL, for four days (NP16-DNA); (C) treated topically with pGTmCMV.IFN-GFP in Dc-chol nanoparticles, 25 μg in 50 μL, for four days (NPDc-DNA). Arrows indicate areas of irritation.

through the stratum corneum, and is more consistent in different mouse models. We demonstrated here that the transgenic IFN-γ-deficient mouse model can be useful for quantitative evaluation of IFN-γ gene expression. However, the overall trend of gene expression was similar to the one observed in CD1 mice. For this reason, for routine formulation screening the CD1 mice would be suitable.

Many previous studies have shown that cationic lipids induce changes in the structure of the DNA, imposing a conformation advantageous for transmembrane delivery of the genetic material. Formation of the $\Psi^-$ form, a more organized highly compacted DNA structure, is important for efficient cellular uptake [26,31]. CD spectra of the NP16-DNA topical formulations indicate significantly higher DNA compaction compared to the NPDc-DNA formulation, characterized by the overall increase of the negative signal [33]. Additional evidence for the increased compaction of the plasmid could be seen by the larger population of smaller particles seen (from both AFM and light scattering results) for the NP16-DNA as compared to the NPDc-DNA formulations. This higher degree of compaction might also contribute to better interaction of the nanoparticles with the skin, and better inter- and intracellular protection of the genetic material, leading to higher protein expression.

The SAXS profile of the NP16-DNA formulation showed a complex polymorphic structure: a dominant lamellar structure and a weaker secondary phase. The relative positions of these peaks follow the geometric progression $\sqrt{3}, \sqrt{8}, \sqrt{11}\ldots$ characteristic of the *Fd3m* cubic phase [34]. The *Fd3m* phase in micellar systems consisted of two

different sizes of inverse micelles, quasi-spherical in nature [35,36]. While this is not a common phase observed in lipid systems [37], it is not unreasonable considering the composition of these systems (the *Fd3m* phase has been observed in another phosphatidylcholine-containing system) [35]. Interestingly, the NPDc-DNA formulation did not show the same structure, but rather appeared to consist of two lamellar phases ($L_a$ and $L_b$). There is evidence, as revealed by electron microscopic visualization of thermostable DNA–cationic lipid complexes, that cationic lipid/DNA complexes organized into lamellar structures could transfect cells efficiently *in vitro* [38]. On the other hand, Zuhorn et al. [39] have shown that the presence of hexagonal phase was crucial for efficient cellular delivery and endosomal escape of the DNA. Foldvari et al. [11] have indicated, based on *in vitro* transfection data, that the efficiency of the gemini surfactants depended on the ability of the system to assume mixed polymorphic (lamellar–cubic–hexagonal) phases. In the present study a direct comparison of the effect of structural properties of nanoparticles could not be made due to the irritation caused by the NPDc-DNA, which would have influenced plasmid delivery into the skin. The importance of the structural features (lamellar–cubic phases) of NP16 nanoparticles *in vivo* will be subjected to further studies.

## 5. Conclusions

Topical treatment with gemini nanoparticles appears to be more effective (compared to naked DNA) and safer (compared to NPDc-DNA) therefore a feasible method for cutaneous non-invasive gene delivery in IFN-γ-deficient mice. Further studies will be needed to correlate the structure – *in vivo* transfection efficiency in order to design highly efficient non-viral gene delivery systems.

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