University of Groningen

An elastic liposomal formulation for RNAi-based topical treatment of skin disorders

Desmet, Eline; Bracke, Stefanie; Forier, Katrien; Taevernier, Lien; Stuart, Marc C. A.; De Spiegeleer, Bart; Raemdonck, Koen; Van Gele, Mireille; Lambert, Jo

Published in:
International Journal of Pharmaceutics

DOI:
[10.1016/j.ijpharm.2016.01.042](https://doi.org/10.1016/j.ijpharm.2016.01.042)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Desmet, E., Bracke, S., Forier, K., Taevernier, L., Stuart, M. C. A., De Spiegeleer, B., Raemdonck, K., Van Gele, M., & Lambert, J. (2016). An elastic liposomal formulation for RNAi-based topical treatment of skin disorders: Proof-of-concept in the treatment of psoriasis. *International Journal of Pharmaceutics*, 500(1-2), 268-274. <https://doi.org/10.1016/j.ijpharm.2016.01.042>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Pharmaceutical nanotechnology

An elastic liposomal formulation for RNAi-based topical treatment of skin disorders: Proof-of-concept in the treatment of psoriasis

Eline Desmet^{a,*}, Stefanie Bracke^a, Katrien Forier^{b,c}, Lien Taevernier^d, Marc C.A. Stuart^e, Bart De Spiegeleer^d, Koen Raemdonck^b, Mireille Van Gele^{a,1}, Jo Lambert^{a,1}

^a Department of Dermatology, Ghent University, Ghent, Belgium

^b Department of Pharmaceutics, Ghent University, Ghent, Belgium

^c Center for Nano and Biophotonics, Ghent University, Ghent, Belgium

^d Department of Pharmaceutical Analysis, Ghent University, Ghent, Belgium

^e Electron microscopy, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

ARTICLE INFO

Article history:

Received 13 November 2015

Received in revised form 15 January 2016

Accepted 16 January 2016

Available online 19 January 2016

Keywords:

Lipid-based nanoparticle

Skin disorder

RNA interference

Liposome

Topical drug delivery

Gene therapy

ABSTRACT

RNA interference (RNAi) is a rapidly emerging approach for targeted gene silencing to alleviate disease pathology. However, lack of efficient carriers for targeted delivery delays the clinical translation of RNAi. An interesting target for local RNAi therapeutics is the skin as it allows direct access to target cells. Still, applications are limited due to the effective skin barrier which hinders penetration. Herein, a description is given of a liposomal carrier, called 'DDC642', capable of delivering RNAi molecules to the epidermis of impaired and intact human skin, without targeting the dermis or circulatory system. In a psoriasis tissue model, down-regulation of the psoriasis marker human beta-defensin 2 by DDC642-delivered siRNA was confirmed, providing proof-of-concept. These liposomes thus hold great potential as topical delivery system for RNAi therapeutics in the treatment of numerous skin diseases.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Since its discovery in mammalian cells, RNA interference (RNAi) has emerged as a powerful gene silencing technology (Elbashir et al., 2001). The strong appeal of RNAi in therapeutics is the potency and specificity of gene expression inhibition. Moreover, the ease of design of RNAi molecules and the versatility of RNAi to target a broad spectrum of mRNAs have made them a promising new class of therapeutics (Bumcrot et al., 2006).

At present, the major hurdle hindering the successful clinical translation of RNAi remains the need for effective and safe vehicles

for targeted RNAi delivery (Chen and Xie, 2012). As the skin allows direct access to target cells, it is an interesting organ for local RNAi delivery. Application of topical or local RNAi therapy is easily confined to the affected area and systemic absorption is significantly less than for other routes of administration (Geusens et al., 2009). Unfortunately, cutaneous drug delivery remains challenging as skin acts as an excellent barrier. Penetration of compounds from outside the body is prevented by this barrier, which resides primarily in the stratum corneum (El Maghraby et al., 2008). Moreover, in vivo delivery of 'naked' RNA molecules is hampered by their susceptibility to degradation by endogenous enzymes. Furthermore, their negative charge, hydrophilic nature and relatively large size (siRNA ~13 kDa) limit their penetration through cell membranes. To overcome these limitations, researchers are investigating penetration enhancing techniques to improve (trans) dermal delivery of RNAi molecules (Geusens et al., 2009). The use of elastic lipid-based vesicles recently gained interest in skin drug delivery (El Maghraby et al., 2008; Pirvu et al., 2010). They differ from conventional liposomes not in morphology but in function. Elastic liposomes are metastable, making them ultra-flexible and highly deformable, enabling penetration through pores much smaller than their own size (Kumar et al., 2012). In the search of highly deformable liposomes, we previously developed

Abbreviations: SEC, SECosomes; HiP, HiPerfect; LPX, lipoplex; DOTAP, 2,3-dioleoyloxy-propyl-trimethylammoniumchloride; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DS, dextran sulphate; KC, keratinocyte; MC, melanocyte; DEFB4, defensin beta 4; hBD-2, human beta-defensin 2.

* Corresponding author at: Department of Dermatology, De Pintelaan 185, Vijverpark, 9000 Ghent, Belgium.

E-mail addresses: eline.desmet@ugent.be (E. Desmet), stefanie.bracke@ugent.be (S. Bracke), katrien.forier@ugent.be (K. Forier), lien.taevernier@ugent.be (L. Taevernier), m.c.a.stuart@rug.nl (M.C.A. Stuart), bart.despiegeleer@ugent.be (B. De Spiegeleer), koen.raemdonck@ugent.be (K. Raemdonck), mireille.vangele@ugent.be (M. Van Gele), jo.lambert@ugent.be (J. Lambert).

¹ Equal contribution.

novel nanosomes called 'SECosomes' (SEC) (Geusens et al., 2010). They are composed of 4 distinct components, namely a cationic lipid (2,3-dioleoyloxy-propyl-trimethylammoniumchloride (DOTAP)), a helper lipid/stabilizer (cholesterol), a single chain surfactant (sodium cholate) and penetration enhancer (30% ethanol). SECosomes have been proven to enable siRNA transfection in cultured primary skin cells and topical delivery in the epidermis of ex vivo human (psoriatic) skin and of a skin-humanized psoriasis mouse model (Bracke et al., 2014; Geusens et al., 2010). However, the efficiency of skin penetration and siRNA internalization into skin's viable cells remained debatable. This research aimed at optimizing the SECosome transfection efficiency and penetration capacity by altering their composition, i.e., surfactant and/or helper lipid, in order to improve their use as a topical delivery system for therapeutic RNAi molecules in the treatment of skin diseases.

2. Materials and methods

2.1. Materials and reagents

DOTAP (2,3-dioleoyloxy-propyl-trimethylammoniumchloride) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Corden Pharma (Liestal, Switzerland). Cholesterol (Chol), sodium cholate (SC), Tween 20/80 (TW20/80) were from Sigma-Aldrich (Bornem, Belgium). Scrambled (non-silencing) siRNA and siRNA against *defensing beta 4* (DEFB4), with or without Cy5-modification, were respectively purchased from Eurogentec (Ougrée, Belgium) and Qiagen Benelux (Antwerp, Belgium). Pre-miR-145, pre-miR negative control #2, miR-203a inhibitor and miRNA inhibitor negative control #1 were purchased from Life Technologies (Ghent, Belgium). HEPES buffer (1 M, HB) was from Sigma-Aldrich (Bornem, Belgium) and diluted to a 20 mM solution (pH 7.4).

Approval from the ethical committee was obtained for the collection of human skin biopsies.

2.2. Particle preparation and complex formation

SECosomes and derivatives (Table 1) were prepared using the solvent evaporation method (Geusens et al., 2010). Lipids were dissolved in chloroform, TW20/80 in sterile distilled water, and SC in >99% EtOH by means of sonication. After mixing the components, solvent was removed by rotary vacuum evaporation above lipid transition temperature. The resulting film was hydrated in HB with 30% EtOH. After overnight incubation, vesicles were extruded 31× through a 100 nm polycarbonate membrane filter (Whatman, Brentfort, UK). Corresponding lipoplexes (LPX) were

prepared by diluting the RNAi molecules with HB to a volume of 100 µL. Liposomes were added under vortex mixing to a liposome/siRNA or antagomiR ratio of 16/1 (w/w), and liposome/pre-miR of 8/1. Average particle size and zeta potential were determined using Zetasizer Nano series (Malvern, Worcestershire, UK).

2.3. Cell culture and transfection procedures

Primary epidermal MCs and KCs were isolated from neonatal foreskin with appropriate informed consent from the donors. MCs were established and cultured as described previously (Naeyaert et al., 1991; Van Gele et al., 2008). KCs were isolated according to Rheinwald and Green and cultured in serum-free keratinocyte growth medium (KGM; Life Technologies, Ghent, Belgium) (Rheinwald and Green, 1975). Cells were housed in an incubator at 37 °C, 99% humidity, and 10% CO₂. 24 h prior to transfection, MCs and KCs were seeded at 5×10^5 cells/well in 6-well flat-bottom cell culture plates. For transfection of psoriasis-induced KCs, cells were seeded at 2×10^5 cells/well in KGM. At 90% confluence, induction of psoriasis-associated features was established as described previously (Bracke et al., 2013). Prior to transfection, cells were washed with PBS and LPXs were added dropwise onto the cells. After 4 h (siRNA, pre-miR) or 24 h (anti-miR) incubation in minimal medium, complexes were removed and medium was replaced. The final concentration of siRNA and pre-miR molecules was 25 nM and 50 nM for anti-miR transfection. HiPerFect (Qiagen, Antwerp, Belgium) was used as a positive control, according to manufacturer's recommendations.

Transfection efficiencies were determined 48 h post-transfection by means of gene silencing, flow cytometry and/or Western blot (as described in Desmet et al., submitted). Transfections were repeated in duplicate or triplicate with 3 different donors.

2.4. Real-time qPCR

Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Antwerp, Belgium) according to manufacturer's requirements. DNase treatment was performed and first-strand cDNA was generated by reverse transcription using iScript cDNA synthesis kit (Bio-Rad, Eke, Belgium). Relative gene expression levels were determined using a SYBR Green I reverse transcription-PCR assay as described by Vandesompele et al. (2002a), with comparative Cq method for quantification. PCR reactions were performed on a MyiQ iCycler (Bio-Rad, Eke, Belgium) using SYBR Green I master mix (Eurogentec, Ougrée, Belgium). Expression levels were normalized using the geometric mean of three reference genes (Vandesompele et al., 2002b).

Table 1

The composition and physicochemical parameters of the liposomes and siRNA-loaded lipoplexes. Values represents the mean $\pm$ SD ($n \geq 3$). PDI: polydispersity index; EE: encapsulation efficiency; I%: internal percentage; P/N: positive to negative charge ratio.

Composition (w)		SEC DOTAP (6) Chol (1) SC (1)	DD61 DOTAP (6) DOPE (1) SC (1)	DD51 DOTAP (5) DOPE (1) SC (1)	DTW2C DOTAP (6) Chol (1) TW20 (1)	DTW8C DOTAP (6) Chol (1) TW80 (1)	DDC642 DOTAP (6) DOPE (4.2) Chol (1.8)
Liposomes	Size (nm)	80.0 $\pm$ 22.8	127.1 $\pm$ 69.0	72.3 $\pm$ 12.9	108.3 $\pm$ 47.9	108.9 $\pm$ 13.2	91.9 $\pm$ 27.5
	PDI	0.28 $\pm$ 0.08	0.28 $\pm$ 0.07	0.27 $\pm$ 0.09	0.18 $\pm$ 0.03	0.24 $\pm$ 0.06	0.22 $\pm$ 0.05
	Zeta (mV)	53.7 $\pm$ 9.1	46.4 $\pm$ 17.3	52.4 $\pm$ 12.0	46.8 $\pm$ 11.6	63.0 $\pm$ 2.5	54.1 $\pm$ 12.3
Lipoplexes (siRNA)	Size (nm)	171.9 $\pm$ 49.0	154.6 $\pm$ 44.4	172.9 $\pm$ 78.1	152.1 $\pm$ 57.2	184.1 $\pm$ 77.4	147.7 $\pm$ 31.9
	PDI	0.28 $\pm$ 0.10	0.29 $\pm$ 0.03	0.36 $\pm$ 0.10	0.40 $\pm$ 0.03	0.40 $\pm$ 0.19	0.21 $\pm$ 0.06
	Zeta (mV)	42.7 $\pm$ 10.6	42.4 $\pm$ 12.2	48.8 $\pm$ 7.9	46.6 $\pm$ 1.7	39.6 $\pm$ 7.7	46.7 $\pm$ 13.4
	EE%	94.8 $\pm$ 0.6	97.9 $\pm$ 1.7	98.0 $\pm$ 1.9	95.6 $\pm$ 3.2	97.5 $\pm$ 0.6	98.6 $\pm$ 1.4
	I%	85.3 $\pm$ 1.7	46.1 $\pm$ 9.4	55.9 $\pm$ 18.8	47.3 $\pm$ 18.4	57.2 $\pm$ 14.6	99.1 $\pm$ 1.1
	P/N ratio	3.94	3.94	3.48	5.41	5.41	3.61

2.5. Skin penetration and therapeutic efficacy

Psoriatic skin biopsies (4 mm) and human excised skin, obtained after informed consent, were cleaned with PBS and used immediately. LPXs containing Cy5-labeled *DEFB4* siRNA ($\pm 5.84 \mu\text{g}$) were applied onto full thickness skin under non-occlusive conditions for 6 h at 4°C. Untreated skin was used as negative control. After embedding in optimal cutting temperature compound, 7 μm cross sections were made and analyzed using a Nikon C1 confocal laser scanning microscope (Nikon Belux, Brussels, Belgium) equipped with Plan fluor 20 $\times$ NA 0.5 objective lens. Images were overlaid (NIS Elements Advanced Research software, Nikon Belux, Brussels, Belgium) on simultaneously collected light transmission images. Processing (brightness/contrast and color balance) was applied equally to all images using Image J software. Experiment was performed in triplicate.

Therapeutic efficacy was analyzed in a psoriatic reconstructed skin model (MatTek, Ashland, Massachusetts). Skin samples were handled according to manufacturer's guidelines and topically treated with LPXs for 48 h ($2.8 \mu\text{g}$ siRNA/cm²). Samples ($n = 2$ per condition) were collected for RNA isolation and qPCR analysis, and for immunohistochemistry.

2.6. Immunohistochemistry

Deparaffinized sections were stained for hBD-2 using Dako immunohistochemistry products (Heverlee, Belgium). Antigens were retrieved using target retrieval solution (pH 6.1) for 30 min. Slides were blocked with EnVision FLEX peroxidase-blocking reagent for 10 min and overnight incubated with primary antibody (Antibodies-online, Aachen, Germany). Detection was performed using the EnVision FLEX/HRP detection reagent and AEC+. Slides were counterstained using EnVision FLEX Haematoxylin and mounted in Aquatex (Merck, Overijse, Belgium). Images were taken using the LEICA DM2000 LED microscope and LAS V4.3 software (Leica, Diegem, Belgium). Staining intensity was determined for 5–10 slides per condition using Image J software.

2.7. Statistical analysis

Statistical analysis of differences between 2 conditions was performed using unpaired *t*-test or nonparametric Mann–Whitney *U* test, and between multiple groups by ANOVA or nonparametric Kruskal–Wallis test using SPSS Statistics 21. A 0.05 level of probability (*P*-value < 0.05) was taken as level of significance.

3. Results

3.1. Vesicle size, zeta potential and encapsulation efficiency

The average vesicle size and surface charge of the different liposomes were characterized by dynamic light scattering (DLS) studies (Table 1). All formulations had a small particle size ranging from ± 72 to ± 127 nm with a narrow size distribution (polydispersity index < 0.29). Zeta potential measurements showed a positive surface charge ranging from ± 46 to 63 mV.

After siRNA complexation (Table 1), size increased for all the formulations (1.2–2.4 times) but remains within the range of previously reported liposomes for medical use (50–400 nm) (Etheridge et al., 2013). The expected decrease in surface charge, by adherence of siRNA to the outer shell of the liposomes, was only significant for the SEC and DTW8C formulations (*P*-values < 0.05). The lack in decrease could be caused by either a low encapsulation efficiency or by the formation of multilamellar structures with the siRNA packed between several vesicle layers (Geusens et al., 2010). A gel retardation study (see Fig. 1 in Desmet et al., submitted)

showed an excellent encapsulation efficiency for all formulations with only a minimal amount of free molecules. In order to evaluate the hypothesis of multilamellar vesicles, anion repulsion assays were conducted, which dissociates surface-bound siRNA from lipoplexes (LPX) by adding competing negatively charged molecules. Incubation with 10-fold (w/w) dextran sulphate (DS) indicated that the majority of siRNA was encapsulated between lipid layers of SEC and DDC642 LPXs, here defined as the internal percentage. The other formulations retain more siRNA on their surface making these molecules vulnerable to degradation (Table 1). Increasing the DS concentration did not influence the release of siRNA (*P*-values > 0.05), indicating that the formulations are stable in anion excess.

3.2. Transfection efficiency

Gene silencing capacity in vitro was evaluated in keratinocytes (KC) and melanocytes (MC), two abundant epidermal cell types, using pre-miR-145 or siRNA against *defensing beta 4* (*DEFB4*) (Fig. 1). MiR-145 was shown to be an invasion suppressor in metastatic melanoma cells, whereas beta-defensin 2 (hBD-2, encoded by *DEFB4*) is upregulated in psoriatic lesions and plays an important role in psoriasis (Dynoodt et al., 2013b; Harder et al., 2004; Hollox et al., 2008; Jansen et al., 2009). Gene silencing capacity of pre-miR-145-loaded LPXs was assessed in MCs by

Fig. 1. Transfection efficiency in MCs transfected with 25 nm pre-miR-145 (A) and in psoriasis-induced KCs using 25 nm *DEFB4* siRNA (B). The expression levels of the target genes FSCN1 (A) and hBD-2 (B) were quantified by real-time qPCR and are relative to that of cells transfected with random sequence pre-miR molecules or scrambled siRNA (100%). Error bars represent the mean $\pm$ SD ($n \geq 6$). *P*-values < 0.05 * significant compared to control, † significant decrease compared to SEC.

examining the expression level of the target gene *fascin homolog 1* (*FSCN1*) (Dynoodt et al., 2013a). Analysis revealed that all formulations were capable of downregulating *FSCN1* (Fig. 1A). Only HiPerfect (HiP), a commercially available transfection reagent, and DDC642 displayed a significantly better silencing capacity than SEC. However, western blot analysis of myosin Va (MyoVa), a second target gene of miR-145, shows only a decrease in protein level when using the DDC642 LPXs (see Fig. 3 in Desmet et al., submitted) (Dynoodt et al., 2013a).

DEFB4 silencing by siRNA was performed in a psoriasis-induced KC model (Bracke et al., 2013). All formulations, except DD51, showed significant silencing of hBD-2 mRNA levels compared to corresponding control samples. However, silencing efficacy was comparable between all formulations (Fig. 1B).

Based on these results, DDC642 was chosen as potential superior delivery carrier and was subjected to a more in-depth analysis.

3.3. In-depth characterization of DDC642

The morphology of the liposomes and their complexes was visualized using cryo-TEM (see Fig. 2 in Desmet et al., submitted). The DDC642 liposomes show typical round-shaped unilamellar structures with a mean size smaller than 100 nm, consistent with DLS measurements (Table 1). After complexation, typical sandwiched lamellar lipoplex structures were observed (Huebner et al., 1999; Weisman et al., 2004). The DDC642 lipoplexes do not have the tendency to aggregate unlike the SECoplexes (Geusens et al., 2010). This phenomenon could be attributed to the stabilizing effect of cholesterol, which was higher in the DDC642 formulation (Lopez-Pinto et al., 2005). The appearance of bulges and non-circular, deformed vesicles indicate the elastic properties and hence the deformable character of the lipoplexes.

Complex formation with microRNA did not significantly alter the physicochemical parameters of the DDC642 LPXs compared to siRNA loading (Table 2). However, miRNA complexation with SEC resulted in significantly smaller lipoplexes, with a similar zeta potential. Gel retardation assays (see Fig. 1 in Desmet et al., submitted) indicated that the SECosomes are inferior for miRNA complexation, which could account for the smaller LPX size and inferior gene silencing capacity compared to their siRNA-containing complexes (Fig. 1). Moreover, SEC miRNA encapsulation appeared to be unstable in the presence of high anion excess (P -value <0.05). For DDC642, miRNA complexation resulted in stable lipoplexes with no significant differences in free or encapsulated miRNA compared to siRNA loading. The stability of the DDC642s can be attributed to the high amount of DOPE, a stabilizer of liposome/DNA complexes, in their formulation (Bozzuto and Molinari, 2015).

The transfection efficiency of antagomiR-containing lipoplexes was examined using an antagomiR directed against miR-203, a psoriasis-specific microRNA highly upregulated in psoriatic plaques (Sonkoly et al., 2007). Only the DDC642 LPXs were able to undermine the suppression effect of miR-203 on SOCS3 (Suppressor of cytokine signaling 3) (Fig. 2A). However, this

Fig. 2. Anti-miR203 transfection in normal KCs. (A) The expression level of the target gene SOCS3 was quantified by real-time qPCR and is relative to that of cells transfected with random sequence anti-miR molecules ($n \geq 9$). (B) SOCS3 protein expression was analyzed via Western blot and quantitative analysis was performed comparing treated versus untreated, control samples (100%). α -Tubulin was used as loading control. Values represent the mean percentage $\pm$ SD ($n = 3$). P -values <0.05 * significant compared to control, † significant compared to SEC.

upregulation could not be confirmed at protein level 48 h post-transfection (Fig. 2B) using a mouse anti-SOCS3 antibody (R&D systems, Abingdon, UK). The superiority of DDC642 over SEC as carrier for microRNA transfection (Figs. 1A and 2A) can be attributed to their high internal percentage (I%), as no differences in the percentage of transfected cells was observed (see Table 1 in Desmet et al., submitted).

3.4. Cytotoxic effects, and physicochemical/biological stability

Cytotoxicity of the liposomes was evaluated both in MCs and in KCs in function of concentration. Cell viability decreased with increasing liposomal concentrations (see Fig. 4A in Desmet et al., submitted). When applying the $20 \mu\text{g mL}^{-1}$ dose on MC, cytotoxicity was significantly higher for the DDC642 liposomes compared to the SECosomes. For KCs, no significant differences could be observed between the different liposomes, but a higher tolerance for DDC642 was noticeable. It is noteworthy that such high concentration were not reached in our in vitro transfection experiments ($<5.32 \mu\text{g mL}^{-1}$, cf. cell viability $\sim 93\%$).

After 30 days of storage at 4°C , The SEC LPXs could be defined as physicochemically stable since no significant differences in vesicle size occurred (see Fig. 4B in Desmet et al., submitted). The DDC642 LPXs on the other hand remained stable in size during a period of 10 days, but increased significantly up to day 20 ($\pm 46\%$). Reassessment of the gene silencing capacity showed that both

Table 2

Physicochemical parameters of the miRNA-loaded lipoplexes. Values represents the mean $\pm$ SD ($n \geq 3$). PDI: polydispersity index; EE: encapsulation efficiency; I%: internal percentage.

		SEC	DDC642
Lipoplexes (miRNA)	Size (nm)	123.2 ± 36.7	131.0 ± 36.8
	PDI	0.34 ± 0.14	0.24 ± 0.05
	Zeta (mV)	47.4 ± 8.6	51.0 ± 6.8
	EE%	98.1 ± 1.2	97.2 ± 4.5
	I%	62.5 ± 2.4	88.3 ± 9.1

formulations retained their biological activity up to 30 days of storage without any decrease in gene silencing capacity (see Fig. 4C in Desmet et al., submitted). In fact, they seemed to perform better after 10 days of storage, with a significant increase in silencing capacity at day 30.

3.5. Penetration capacity and therapeutic efficacy

The penetration capacity of the lipoplexes was investigated in excised human skin after treatment with Cy5-labeled siRNA-

loaded complexes. Fig. 3A shows confocal fluorescence/transmission electron microscopy overlay images of a cross-section of treated normal skin. SEC (I) lipoplexes are mainly confined to the stratum corneum, whereas DDC642 LPXs (II) penetrate the different strata of the epidermis. In lesional psoriatic skin, which has an impaired skin barrier, the SECoplexes are also able to penetrate all the epidermal layers (Fig. 3B).

Treatment of normal human skin (intact or tape-stripped) in a Franz diffusion cell set-up (Desmet et al., submitted) generated no detectable Cy5 signal in the receptor compartments after 6 h,

Fig. 3. Skin penetration capacity and therapeutic efficacy of DDC642 LPXs. (A, B) Representative confocal images from skin biopsy cross sections, both of normal (A) and lesional psoriatic skin (B) after 6 h incubation with Cy5-siRNA complexes of SEC (I) and DDC642 (II). Migration of the complexes into the skin strata can be tracked by means of the red fluorescent signal derived from Cy5-emission. Scale bar: 100 nm. (C) Therapeutic efficacy of DEFBA siRNA-containing DDC642 lipoplexes in an ex vivo psoriatic tissue model. After 48 h of treatment, a significant decrease in hBD-2 was noticeable for treated (II) versus untreated (I) tissue samples. Scale bar: 500 nm.

indicating that the siRNA-containing lipoplexes do not or only minimally (<50 pm) penetrate the dermis (data not shown).

By evaluating the down-regulation of hBD-2 in a psoriatic tissue model, the therapeutic efficacy of *DEFB4* siRNA-containing DDC642 LPXs was validated. Forty-eight hours post-treatment, hBD-2 mRNA expression decreased to ~70% of untreated control levels (data not shown) and a significant decrease in protein expression was apparent (Fig. 3C, *P*-value <0.05). These results indicate that the siRNA delivered to the epidermis still maintains its silencing function and thus proves the therapeutic efficacy of the DDC642 lipoplexes.

4. Discussion

RNA interference represents a promising therapeutic strategy due to its potency and specificity of gene expression inhibition. However, RNAi therapies are hindered by disadvantageous intrinsic properties of small RNA molecules and their susceptibility to degradation. An effective carrier for the delivery of these molecules to their target cells is therefore crucial in the development of targeted RNAi delivery (4). Among the nanoparticles currently being investigated, lipid carriers preside because of obvious advantages of high degree of biocompatibility, biodegradability and versatility. The proven safety and efficacy of lipid-based carriers make them attractive candidates for the formulation of pharmaceuticals (Attama et al., 2012; Pradhan et al., 2013).

For topical applications, elastic lipid-based liposomes recently gained interest in order to overcome the skin barrier and improve skin drug delivery, as they are ultraflexible and highly deformable enabling penetration through small pores (El Maghraby et al., 2008; Kumar et al., 2012; Pirvu et al., 2010). Recently, our group developed the ultradeformable SECosomes which contains an edge activator and penetration enhancer. In order to further improve their penetration and transfection ability, we modified their composition while respecting several rules and principles: First, DOTAP remained the main component. DOTAP holds a positive (+1) electrical charge on the trimethylammonium propane (TAP) head group, ideal for interaction with negatively charged nucleic acids (Kim et al., 2004). However, 100% DOTAP is inefficient for gene delivery and requires helper lipids such as DOPE, DOPC (dioleoyl-phosphatidyl-choline) and cholesterol (Balazs and Godbey, 2011; Kim et al., 2001; Zuidam and Barenholz, 1998). DOPE as helper lipid was examined at 6/1 (w/w) and 5/1 ratio DOTAP/DOPE as previously proven to generate effective transfection agents (Geusens et al., 2010; Kim et al., 2001). The combination of cholesterol and DOPE in one formulation, first described by Kim et al. in the development of a nonviral vector for gene transfer to human ovarian adenocarcinoma cells, was also evaluated (Kim et al., 2003, 2002). Second, single chain surfactants act as so-called edge activators destabilizing the lipid bilayers and providing greater elasticity compared to conventional liposomes (Cevc, 1996; El Maghraby et al., 2008). However, the choice of edge activator plays an important role in the (trans) dermal delivery of nucleic acids, and thus the use of Tween 20 or Tween 80 instead of sodium cholate has been investigated (Bragagni et al., 2012; Elsayed et al., 2007; Ita et al., 2007; Lee et al., 2005). Finally, all formulations contained 30% ethanol as a penetration enhancer (Touitou et al., 2000).

This experimental set-up lead to the discovery of a superior liposome, called 'DDC642'. Compared to the SEC, the DDC642 liposomes form stable complexes with both siRNA and miRNA molecules, and enable a high transfection of cultured primary skin cells. Moreover, they deliver functional RNAi molecules to the different epidermal layers of normal or impaired skin, without crossing the dermal compartment, whereas the

SECoplexes remain restricted to the stratum corneum in non-impaired skin. The unique combination of DOTAP, DOPE, cholesterol and EtOH can be held accountable for the creation of flexible yet stable liposomes with the capacity to penetrate the epidermis. DOTAP exhibits the ability to induce phase reorganization in lipids systems containing anionic phospholipids, such as the skin and endosomal membranes. This reorganization is promoted by DOPE to facilitate fusion with or destabilization of target membranes (Hafez et al., 2001; Ulrich, 2002; Xu and Szoka, 1996). The addition of cholesterol increases liposomal stability, reduces aggregate formation and prevents hydrolysis and leakage of the encapsulated drug (Mozafari, 2007; Simon and McIntosh, 1986; Socaciu et al., 2000), whereas ethanol fluidizes lipid membranes and creates more flexible liposomes (Ghanbarzadeh and Arami, 2013; Touitou et al., 2000). By combining these aspects into one formulation, a suitable carrier was created for the delivery of RNAi molecules to human epidermis, without requirement of additional penetration enhancement techniques or expensive Accell self-delivering siRNA (Benson, 2005; Gonzalez-Gonzalez et al., 2010; Hegde et al., 2014; Hickerson et al., 2013). This makes them a suitable topical carrier system in the treatment of various skin diseases, be they with or without skin barrier defect. Here, we took psoriasis as a model disease. Moreover, if transdermal bioavailability is desired, 'DDC642' could be modified to further enhance skin penetration by e.g., the addition of skin penetrating peptides, such as trans-activating transcriptional activator (TAT), polyarginine or SPACE-peptide (skin penetrating and cell entering peptide) (Chen et al., 2014; Desai et al., 2010; Hsu and Mitragotri, 2011; Kamada et al., 2007; Rothbard et al., 2000; Sawant and Torchilin, 2010).

5. Conclusion

In this study, liposomes are formed by the unique blend of DOTAP, DOPE, cholesterol and EtOH, resulting in flexible liposomes with the ability to penetrate human epidermis with or without barrier defect. These 'DDC642' liposomes have been shown to be effective carriers for RNAi delivery in cultured primary skin cells and in a psoriasis tissue model in vitro. Future research will focus on the therapeutic efficacy of DDC642 liposomes loaded with RNAi molecules against anti-psoriasis targets, in order to present a topical anti-psoriasis therapy. As a consequence, this carrier could aid in improving the current scarcity of FDA-approved topical RNAi therapies and drugs.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgements

We thank Els Van Maelsaeke, Martine De Mil and Marie-Chantal Herteleer for their technical assistance. This research was funded by a BOF-project (number 01J10211T, Ghent University, Belgium) and TGO grant (number 120829, IWT, Belgium).

References

- Attama, A., Momoh, M., Builders, P., 2012. Lipid nanoparticle drug delivery systems: a revolution in dosage form design and development. In: Sezer, A. (Ed.), *Recent Advances in Novel Drug Carrier Systems*. InTech, Rijeka doi:<http://dx.doi.org/10.5772/50486>.
- Balazs, D.A., Godbey, W., 2011. Liposomes for use in gene delivery. *J. Drug Deliv.* 2011 doi:<http://dx.doi.org/10.1155/2011/326497>.
- Benson, H.A., 2005. Transdermal drug delivery: penetration enhancement techniques. *Curr. Drug Deliv.* 2, 23–33.
- Bozzuto, G., Molinari, A., 2015. Liposomes as nanomedical devices. *Int. J. Nanomed.* 10, 975–999.

- Bragagni, M., Mennini, N., Maestrelli, F., et al., 2012. Comparative study of liposomes, transfersomes and ethosomes as carriers for improving topical delivery of celecoxib. *Drug Deliv.* 19, 354–361.
- Bracke, S., Desmet, E., Guerrero-Aspizua, S., et al., 2013. Identifying targets for topical RNAi therapeutics in psoriasis: assessment of a new in vitro psoriasis model. *Arch. Dermatol. Res.* 305, 501–512.
- Bracke, S., Carretero, M., Guerrero-Aspizua, S., et al., 2014. Targeted silencing of DEFB4 in a bioengineered skin-humanized mouse model for psoriasis: development of siRNA SECosome-based novel therapies. *Exp. Dermatol.* 23, 199–201.
- Bumcrot, D., Manoharan, M., Kotliansky, V., et al., 2006. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat. Chem. Biol.* 2, 711–719.
- Cevc, G., 1996. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. *Crit. Rev. Ther. Drug Carrier Syst.* 13, 257–388.
- Chen, J., Xie, J., 2012. Progress on RNAi-based molecular medicines. *Int. J. Nanomed.* 7, 3971–3980.
- Chen, M., Zakrewsky, M., Gupta, V., et al., 2014. Topical delivery of siRNA into skin using SPACE-peptide carriers. *J. Control. Release* 179, 33–41.
- Desai, P., Patilola, R.R., Singh, M., 2010. Interaction of nanoparticles and cell-penetrating peptides with skin for transdermal drug delivery. *Mol. Membr. Biol.* 27, 247–259.
- Desmet, E., Bracke, S., Forier, K., et al. (submitted). In-depth characterization of the topical liposomal carrier DDC642. Data in Brief.
- Dynodot, P., Mestdagh, P., Van Peer, G., et al., 2013a. Identification of miR-145 as a key regulator of the pigmentary process. *J. Invest. Dermatol.* 133, 201–209.
- Dynodot, P., Speckaert, R., De Wever, O., et al., 2013b. miR-145 overexpression suppresses the migration and invasion of metastatic melanoma cells. *Int. J. Oncol.* 42, 1443–1451.
- Elbashir, S.M., Harborth, J., Lendeckel, W., et al., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498.
- Elsayed, M.M., Abdallah, O.Y., Naggar, V.F., et al., 2007. Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen. *Pharmazie* 62, 133–137.
- El Maghraby, G.M., Barry, B.W., Williams, A.C., 2008. Liposomes and skin: from drug delivery to model membranes. *Eur. J. Pharm. Sci.* 34, 203–222.
- Etheridge, M.L., Campbell, S.A., Erdman, A.G., et al., 2013. The big picture on nanomedicine: the state of investigational and approved nanomedicine products. *Nanomedicine* 9, 1–14.
- Geusens, B., Sanders, N., Prow, T., et al., 2009. Cutaneous short-interfering RNA therapy. *Expert Opin. Drug Deliv.* 6, 1333–1349.
- Geusens, B., Van Gele, M., Braat, S., et al., 2010. Flexible nanosomes (SECosomes) enable efficient siRNA delivery in cultured primary skin cells and in the viable epidermis of ex vivo human skin. *Adv. Funct. Mater.* 20, 4077–4090.
- Ghanbarzadeh, S., Arami, S., 2013. Enhanced transdermal delivery of diclofenac sodium via conventional liposomes, ethosomes, and transfersomes. *Biomed. Res. Int.* 2013 doi:http://dx.doi.org/10.1155/2013/616810.
- Gonzalez-Gonzalez, E., Speaker, T.J., Hickerson, R.P., et al., 2010. Silencing of reporter gene expression in skin using siRNAs and expression of plasmid DNA delivered by a soluble protrusion array device (PAD). *Mol. Ther.* 18, 1667–1674.
- Hafez, I.M., Maurer, N., Cullis, P.R., 2001. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.* 8, 1188–1196.
- Harder, J., Meyer-Hoffert, U., Wehkamp, K., et al., 2004. Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. *J. Invest. Dermatol.* 123, 522–529.
- Hegde, V., Hickerson, R.P., Nainamalai, S., et al., 2014. In vivo gene silencing following non-invasive siRNA delivery into the skin using a novel topical formulation. *J. Control. Release* 196, 355–362.
- Hickerson, R.P., Wey, W.C., Rimm, D.L., et al., 2013. Gene silencing in skin after deposition of self-delivery siRNA with a motorized microneedle array device. *Mol. Ther. Nucleic Acids* 2 doi:http://dx.doi.org/10.1038/mtna.2013.56.
- Hollox, E.J., Huffmeier, U., Zeeuwen, P.L., et al., 2008. Psoriasis is associated with increased beta-defensin genomic copy number. *Nat. Genet.* 40, 23–25.
- Hsu, T., Mitragotri, S., 2011. Delivery of siRNA and other macromolecules into skin and cells using a peptide enhancer. *Proc. Natl. Acad. Sci. U. S. A.* 108, 15816–15821.
- Huebner, S., Battersby, B.J., Grimm, R., et al., 1999. Lipid–DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy. *Biophys. J.* 76, 3158–3166.
- Ita, K.B., Du Preez, J., Lane, M.E., et al., 2007. Dermal delivery of selected hydrophilic drugs from elastic liposomes: effect of phospholipid formulation and surfactants. *J. Pharm. Pharmacol.* 59, 1215–1222.
- Jansen, P.A., Rodijk-Olthuis, D., Hollox, E.J., et al., 2009. Beta-defensin-2 protein is a serum biomarker for disease activity in psoriasis and reaches biologically relevant concentrations in lesional skin. *PLoS One* 4 doi:http://dx.doi.org/10.1371/journal.pone.0004725.
- Kim, T.W., Chung, H., Kwon, I.C., et al., 2001. Optimization of lipid composition in cationic emulsion as in vitro and in vivo transfection agents. *Pharm. Res.* 18, 54–60.
- Kim, C.K., Haider, K.H., Choi, S.H., et al., 2002. Nonviral vector for efficient gene transfer to human ovarian adenocarcinoma cells. *Gynecol. Oncol.* 84, 85–93.
- Kim, C.K., Choi, E.J., Choi, S.H., et al., 2003. Enhanced p53 gene transfer to human ovarian cancer cells using the cationic nonviral vector, DDC. *Gynecol. Oncol.* 90, 265–272.
- Kim, A., Lee, E.H., Choi, S.H., et al., 2004. In vitro and in vivo transfection efficiency of a novel ultra-deformable cationic liposome. *Biomaterials* 25, 305–313.
- Kamada, H., Okamoto, T., Kawamura, M., et al., 2007. Creation of novel cell-penetrating peptides for intracellular drug delivery using systematic phage display technology originated from TAT transduction domain. *Biol. Pharm. Bull.* 30, 218–223.
- Kumar, R., Rana, A.C., Bala, R., et al., 2012. Formulation and evaluation of elastic liposomes of clotrimazole. *IJDDR* 4, 348–355.
- Lee, E.H., Kim, A., Oh, Y.K., et al., 2005. Effect of edge activators on the formation and transfection efficiency of ultra-deformable liposomes. *Biomaterials* 26, 205–210.
- Lopez-Pinto, J.M., Gonzalez-Rodriguez, M.L., Rabasco, A.M., 2005. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *Int. J. Pharm.* 298, 1–12.
- Mozafari, M.R., 2007. *Nanomaterials and Nanosystems for Biomedical Applications*. Springer, Dordrecht 162 pp.
- Naeyaert, J.M., Eller, M., Gordon, P.R., et al., 1991. Pigment content of cultured human melanocytes does not correlate with tyrosinase message level. *Br. J. Dermatol.* 125, 297–303.
- Pirvu, C.D., Hlevca, C., Ortan, A., et al., 2010. Elastic vesicles as drugs carriers through the skin. *Farmacia* 58, 128–135.
- Pradhan, M., Singh, D., Singh, M.R., 2013. Novel colloidal carriers for psoriasis: current issues, mechanistic insight and novel delivery approaches. *J. Control. Release* 170, 380–395.
- Rheinwald, J.G., Green, H., 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell. Mol. Biol. Lett.* 6, 331–343.
- Rothbard, J.B., Garlington, S., Lin, Q., et al., 2000. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat. Med.* 6, 1253–1257.
- Simon, S.A., McIntosh, T.J., 1986. Depth of water penetration into lipid bilayers. *Methods Enzymol.* 127, 511–521.
- Sawant, R., Torchilin, V., 2010. Intracellular transduction using cell-penetrating peptides. *Mol. Biosyst.* 6, 628–640.
- Socaciu, C., Jessel, R., Diehl, H.A., 2000. Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy. *Chem. Phys. Lipids* 106, 79–88.
- Sonkoly, E., Wei, T., Janson, P.C., et al., 2007. MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? *PLoS One* 2 doi:http://dx.doi.org/10.1371/journal.pone.0000610.
- Touitou, E., Dayan, N., Bergelson, L., et al., 2000. Ethosomes—novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. *J. Control. Release* 65, 403–418.
- Ulrich, A.S., 2002. Biophysical aspects of using liposomes as delivery vehicles. *Biosci. Rep.* 22, 129–150.
- Vandesompele, J., De Paepe, A., Speleman, F., 2002a. Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR. *Anal. Biochem.* 303, 95–98.
- Vandesompele, J., De Preter, K., Pattyn, F., et al., 2002b. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, 1–11.
- Van Gele, M., Geusens, B., Schmitt, A.M., et al., 2008. Knockdown of myosin Va isoforms by RNAi as a tool to block melanosome transport in primary human melanocytes. *J. Invest. Dermatol.* 128, 2474–2484.
- Weisman, S., Hirsch-Lerner, D., Barenholz, Y., et al., 2004. Nanostructure of cationic lipid–oligonucleotide complexes. *Biophys. J.* 87, 609–614.
- Xu, Y., Szoka Jr., F.C., 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 35, 5616–5623.
- Zuidam, N.J., Barenholz, Y., 1998. Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. *Biochim. Biophys. Acta* 1368, 115–128.