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Development of edge-activated liposomes for siRNA delivery to human basal epidermis for melanoma therapy

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ABSTRACT

Delivery of macromolecules such as siRNA into cells that reside in the basal epidermis of the skin is a major challenge due to the transport barriers that need to be overcome. siRNAs have potential therapeutic applications in various dermatological diseases such as psoriasis, atopic dermatitis, and cancer. Unfortunately, a low permeability of siRNA through the stratum corneum and epidermis has significantly limited its use for topical application. The *objective* of this study was to develop a topical siRNA delivery system that can permeate through the stratum corneum and viable epidermis and efficiently deposit therapeutic levels of siRNA to the basal epidermis/upper dermis where melanoma cells reside. To achieve this objective, a series of liposome compositions that contained various concentrations of edge activator in their structures were prepared and then complexed with siRNA at different ratios to generate a small library of liposome-siRNA complexes (lipoplexes) with different physicochemical properties. In this study we used melanoma as a disease model. Through use of quantitative imaging analysis, we identified the necessary design parameters for effective permeation of lipoplexes through the skin layers and deposition at the upper dermis. The ability of the formulated lipoplexes to internalize into melanoma cells, knockdown the expression of the BRAF protein and induce cell death in melanoma cells was studied by fluorescent microscopy, in-cell immunofluorescence assay and WST-1 cell proliferation assay. By providing direct quantitative and qualitative microscopy evidence, the results of this study demonstrate for the first time that the passive delivery of an edge-activated liposomal formulation can effectively carry siRNA through the stratum corneum and deposit it at the lower epidermis/upper dermis.

1. Introduction

Melanoma is the most malignant form of skin cancer and is a lead cause for the majority of skin cancer deaths worldwide. Over the past decades, some progress has been made in the treatment of melanoma using immunotherapy, chemotherapy and radiotherapy [1]; however, these conventional treatment methods have produced limited success due to significant immune related toxicities, frequent development of drug resistance and poor drug tissue distribution. Therefore, there is a significant need for alternative approaches of drug delivery to the melanoma cells.

One recent successful treatment approach includes the localized drug delivery to melanoma cells via the topical route [2,3]. For melanoma therapy, delivery of therapeutics to upper layers of the dermis is critical as melanocytes reside in the basal epidermis and on the top of dermis [4]. Therefore, drug molecules need to pass not only through the stratum corneum but also across the viable epidermis in order to reach the melanocytes. Due to the small pore sizes, the stratum corneum is a significant barrier especially for the delivery of macromolecules such as siRNA and plasmid DNA (pDNA) [5]. In the past decade a few studies have shown progress in delivering macromolecules such as siRNA to the upper epidermis by using lipids [2,6]. However, none of the lipid-based systems has been able to provide a direct evidence to show the effective permeation through stratum corneum and deposition of macromolecules deep in the basal epidermis. For example, Geusens et al. (2009), developed the so-called ultradeformable liposomes for siRNA delivery to melanoma cells which could efficiently transfect cells [2]. However, the ability of the developed liposomal system in permeating skin layers was not demonstrated. Later, the same group in 2010 demonstrated that the developed ultradeformable liposomal system can penetrate stratum corneum and deposit siRNA at the upper epidermis but did not reach lower epidermis/upper dermis where melanocytes reside [6]. This prompted us to take upon this task and develop a liposomal system that can effectively reach and deposit siRNA at lower epidermis/upper dermis. Therefore, the *objective* of this

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study was to develop a topical siRNA delivery system that can permeate through the stratum corneum and viable epidermis and efficiently deliver BRAF-targeted siRNA to the basal epidermis where melanoma cells reside. In this study, we focused on designing a delivery system for topical administration of BRAF-siRNA (v-Raf murine sarcoma viral oncogene homolog B). Molecular analysis of melanoma cells from patients has shown that the majority of the melanocytes contain a mutation in the gene that encodes BRAF protein. Among BRAF mutations, 90% involves a single point mutation that substitutes thymine with adenine at nucleotide 1799 [7]. This mutation in the gene encoding BRAF protein activates the downstream signals of the mitogen-activated protein kinase pathway and ultimately causes an oncogenic increase of melanocytes proliferation and division [8]. Due to the high incidence of mutation in BRAF gene in melanoma cells and increased risk of mortality, we selected the BRAF-siRNA as the model therapeutic for delivery.

Our group has previously shown that cationic moieties should be present in a molecule to effectively permeate through the stratum corneum and deposit in the skin [9]. Therefore, to achieve the objective and in an attempt to overcome the stratum corneum barrier we first prepared a series of cationic liposomal formulations equipped with sodium cholate (NaChol) as an edge-activator. While other edge-activators such as ethanol, sodium deoxycholate and Tween exist, we utilized NaChol due to its higher activity [6,10]. NaChol is a surfactant that is known for its ability to open pores in stratum corneum accelerating permeation of nanoparticles through the skin. This molecule has a pKa of 5.5 which is close to the pH of skin. It has been suggested that at this pH, NaChol protonation occurs in the lipid complex. This protonation tends to be strongly exothermic and this may modulate the skin barrier in favor of the lipoplex permeation [6]. Next, through the use of quantitative imaging analysis we identified the necessary design parameters for effective permeation of liposome-siRNA complexes (lipoplexes) through the skin layers and deposition in the upper dermis. The liposomal formulations were then examined in terms of their ability to internalize into melanoma cells by quantitative fluorescent microscopy and effective knockdown of BRAF expression by in-cell immunofluorescence assay. Finally, the ability of the lipoplexes to kill melanoma cells was studied by using WST-1 cell proliferation assay.

2. Materials and methods

2.1. Preparation of liposomes

Liposomes were prepared by a solvent dispersion technique as previously described [11]. Briefly, 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) (Avanti Polar Lipids Inc., AL, USA) at the concentration of 10 mg/ml was dissolved in chloroform (Sigma Aldrich, USA) at room temperature. Using ultra-sonication, sodium cholate (NaChol) (Sigma Aldrich, USA) at the concentration of 10 mg/ml was dissolved in ethanol (>99.5%) (Sigma Aldrich, USA). DOTAP solution was first mixed with NaChol solution at weight:weight (w:w) ratios of 1:4, 1:6, 1:8, and 1:10 (NaChol: DOTAP) and then the solvent was evaporated to a thin film layer using a rotary evaporator. The solvent trace was evaporated by N_2 streaming. The lipid film was hydrated in HEPES buffer (100 mM, pH 7.4) for 24 h at room temperature to let the liposomes assemble. Liposomes were then extruded gradually at room temperature through a polycarbonate filter with 100 nm pore size using an extruder (Northern Lipids, Inc., Vancouver, BC, Canada). To obtain fluorescently labeled liposomes for skin permeation and intracellular localization experiments, DOTAP was mixed with green fluorescently labeled DOTAP (DOTAP-NBD) (Avanti Polar Lipids Inc., AL, USA) at 100:1 mol/mol ratio and the process was repeated as above. Others have also reported the use of DOTAP and NBD-DOTAP at 100:1 mol/mol ratio for the preparation of green fluorescent liposomes [12].

2.2. Preparation of liposome-siRNA complexes

Lipoplexes were prepared as previously described [12,13]. Briefly, siRNA was dissolved in nuclease free water. Then, the prepared liposomes were mixed with siRNA solution at w:w ratios of 4:1, 8:1, 12:1 and 16:1 (liposome:siRNA) and incubated at room temperature for 30 min. To prepare, dual labeled lipoplexes, green fluorescently labeled liposomes were mixed with red fluorescently labeled siRNA (siGLO) (Dharmacon Inc., CO, USA).

2.3. Particle size and zeta potential analysis

Liposomes and lipoplexes were prepared as mentioned above and the average size and zeta potential of liposomes before and after complexation with siRNA were measured by dynamic light scattering and laser Doppler Velocimetry (Nano-ZS Zetasizer, Malvern Instruments, U.K.), respectively. Three independent batches of liposomes and lipoplexes in HEPES buffer (20 mM, pH 7.4) were prepared and 15 measurements from each sample were made. Data are reported as means ± s.d. ($n = 3$).

2.4. siRNA charge neutralization study by gel retardation assay

The neutralization of negative charges in siRNA by positively charged liposomes was studied by gel retardation assay. Free siRNA and liposome-siRNA complexes were electrophoresed on 4% agarose gel containing 0.5% ethidium bromide at 150v for 1 h. The gel was visualized under a UV light using Gel Documentation System 920 (NucleoTech, San Mateo, CA, USA).

2.5. Skin permeation study

The skin permeation studies of fluorescently labeled lipoplexes were performed using dermatomed freshly excised human cadaver skin from a single donor, the posterior leg of a 54 years old male (New York Firefighters Skin Bank, NY, NY). The skin samples were cut into appropriate sizes and mounted on vertical glass Franz diffusion cells (Permegear, Inc., Hellertown, PA) with a receptor volume of 5.0 ml and donor area of 0.64 cm^2. At the time of the experiment, frozen skin samples were cut into pieces with desired sizes, slowly thawed and hydrated in filtered phosphate buffer saline (PBS) pH = 7.4 for 15 min. Then, the skin samples were gently mounted on Franz diffusion cells in such a way that the dermis was in contact with the receptor. The receptor compartment of each cell was filled with filtered PBS and maintained at 37 °C under synchronous continuous stirring using a magnetic stirrer, whereas the skin surface temperature was unoccluded and maintained at ambient room temperature. As part of our quality control, before each experiment the skin integrity was tested by measuring the skin conductivity [14]. Skin pieces with integrity below 1.00 mS/cm were considered as “damaged” and replaced with another skin piece with acceptable resistance [15]. Each formulation was studied with 3 replicates. At time zero, 100 μl of each sample containing 3 μg siRNA was added to the donor compartment of Franz diffusion cell using a pipette set. As the control groups, 100 μl of phosphate buffer saline (PBS) with pH 7.4 was added to the donor compartment of Franz diffusion cell (3 replicates). The skin surface was left uncovered for uniform drying of the formulation on skin surface. After 24 h, the remaining solution was collected and washed thoroughly off the skin surface with distilled water. The entire receptor of the cells was collected and tested for fluorescent intensity using a fluorescence spectrophotometer (Hitachi Hi-Tech, Tokyo, Japan). All skin samples were collected and studied by fluorescent microscopy.

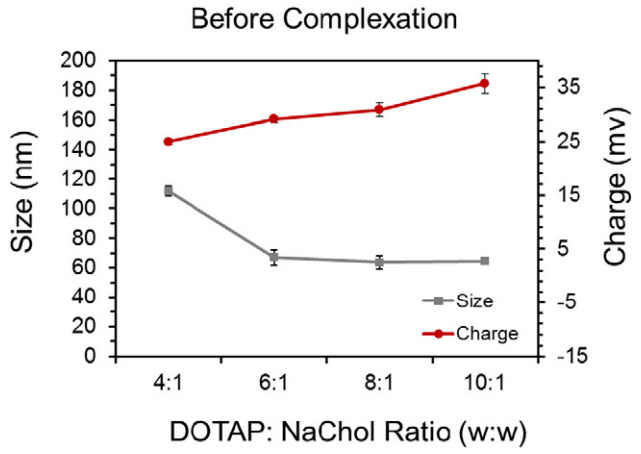

Fig. 1. Size and zeta potential analysis of empty liposomes at different DOTAP to NaChol ratios ranging from 4:1 to 10:1 (w:w). Data are reported as means ± s.d. (n = 3).

2.6. Fluorescent microscopy imaging of skin samples

The collected skin samples were immediately fixed with formaldehyde 10% and incubated overnight at 4 °C. Then, the samples were frozen in OCT embedding media (Sakura Finetek, CA, USA) and sectioned at a thickness of 20 μm using a cryotome (Leica CM1850, Nussloch, Germany). Skin cuts were laid on slides, washed with distilled water and dried at room temperature in the dark. One drop of Fluoromount Aqueous Mounting Medium (Sigma Aldrich, USA) was added to the slide which was then covered with a glass cover slip. Skin samples were imaged using a fluorescent microscope (Olympus, Center Valley, PA, USA) to evaluate the depth of lipoplex permeation. The percentage of lipoplexes in each skin layer was quantified by using ImageJ NIH software (www.imagej.nih.gov). To quantify the concentration of the siGLO that permeated into each skin layer, a standard curve was first plotted by measuring the fluorescent intensity of different concentrations of

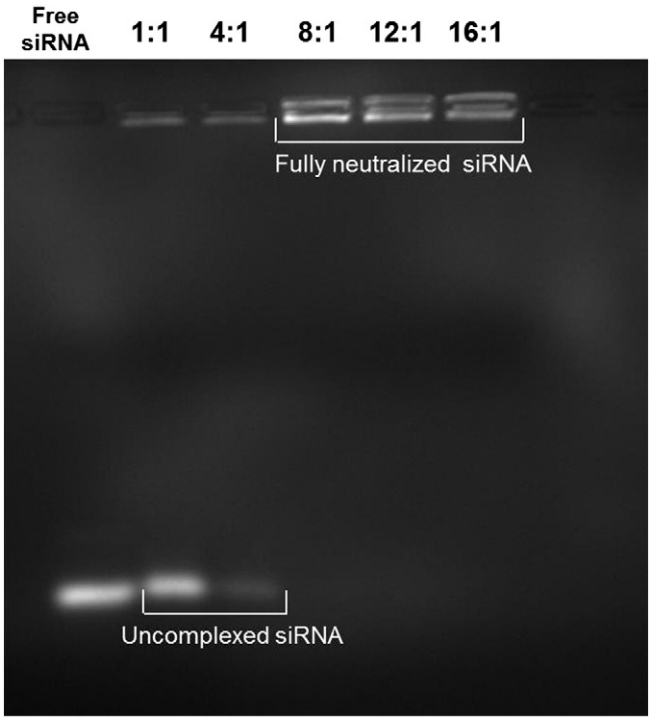

Fig. 3. Gel retardation assay of liposome-siRNA complexes. DOTAP:NaChol (8:1) was complexed with siRNA at different ratios ranging from 1:1 to 16:1 (w:w) followed by visualization of siRNA mobility on the agarose gel. Naked siRNA was used as control.

liposome-siGLO complexes using a fluorescence spectrophotometer F7000 (Hitachi Hi-Tech, Tokyo, Japan). The absorption/emission wavelengths of the instrument were set at 557/570 nm, respectively. Linearity was studied in the range of 0.046–3.0 μg/100 μl of siGLO with each concentration being assayed in triplicate. Linearity was observed in

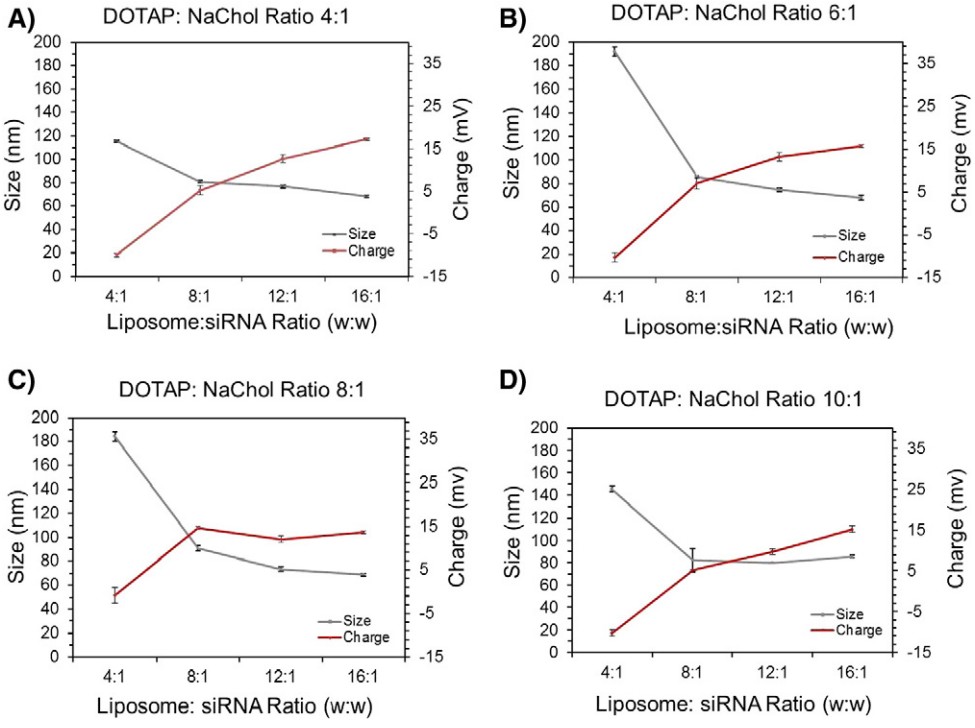

Fig. 2. Size and zeta potential analysis of liposome-siRNA complexes at different w:w ratios ranging from 4:1 to 16:1. Data are reported as means ± s.d. (n = 3).

this range with an R^2 value of 0.9929. The stratum corneum layers of the skin samples were first separated from the skin by tape stripping. Then, the epidermis was separated from the dermis mechanically by using a tweezer. The liposome-siGLO complexes in the stratum corneum, epidermis and dermis were then extracted in PBS pH 7.4 by homogenization (BeadBug™ Microtube Homogenizer, Edison, NJ, USA). The concentration of the permeated siGLO into each layer of the skin was then calculated using the standard curve.

2.7. Evaluation of intracellular localization of liposome-siRNA complexes

To examine the cellular internalization of lipoplexes, UACC-903 melanoma cells were seeded at a density of 2000 cells per well in a 24-well plate and incubated overnight at 37 °C. The next day, green fluorescently labeled liposomes were mixed with 20 μM of siGLO (red fluorescence labeled scrambled siRNA) solution at the w:w ratio of 16:1 (liposome: siGLO) and incubated at room temperature for 30 min before cell transfection. Cells were transfected with the liposome-siGLO complexes for 1 h at 37 °C. After 1 h incubation, cells were washed 3–4 times using Dulbecco Phosphate Buffer Saline (DPBS) and then the cell nuclei were stained by adding 6 μl of DAPI (blue fluorescent dye) (Life Technologies, NY, USA) followed by incubation for another 20 min. Finally, the cells were washed 3–4 times with DPBS and visualized using a fluorescent microscope.

2.8. Evaluation of BRAF expression knockdown in melanoma cells

To investigate the ability of the developed liposomal system to deliver BRAF siRNA into the melanoma cells, an in-cell immunofluorescence technique was used to visualize the expression of BRAF inside the cells.

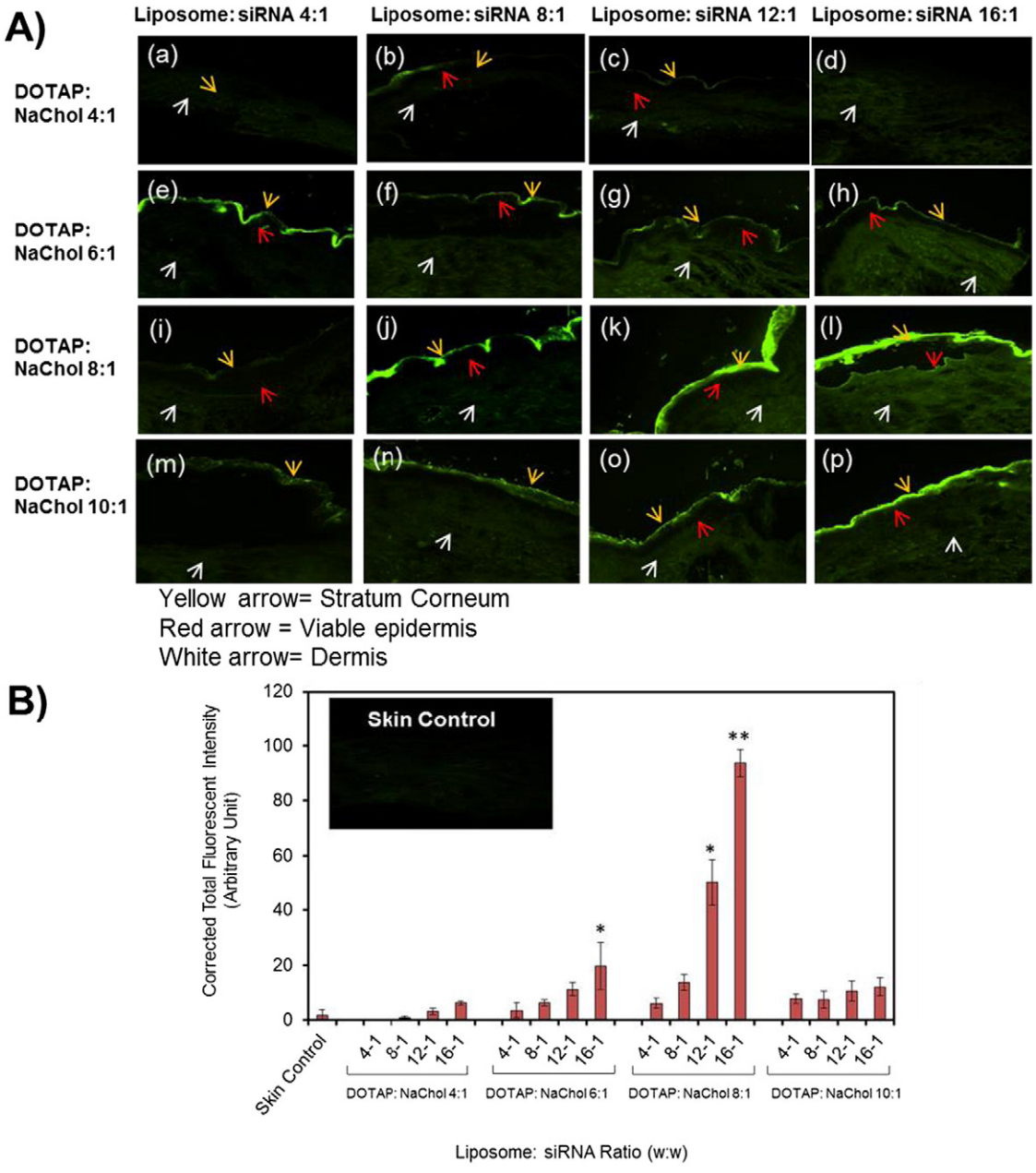

Fig. 4. A) Fluorescent microscopy images of liposomes (green fluorescently labeled) in complex with siRNA that permeated through the skin layers. a-d) liposome:siRNA (Liposome:NaChol 4:1 ratio) complexes at w:w ratios of 4:1, 8:1, 12:1, 16:1, respectively. e–h) liposome:siRNA (Liposome:NaChol 6:1 ratio) complexes at w:w ratios of 4:1, 8:1, 12:1, 16:1, respectively. i–l): liposome:siRNA (Liposome:NaChol 8:1 ratio) complexes at w:w ratios of 4:1, 8:1, 12:1, 16:1, respectively. m–p): liposome:siRNA (Liposome:NaChol 10:1 ratio) complexes at w:w ratios of 4:1, 8:1, 12:1, 16:1, respectively. B) Quantification of fluorescent intensity of the deposited lipoplexes in the skin. Data are reported as means ± s.d. (n = 3).

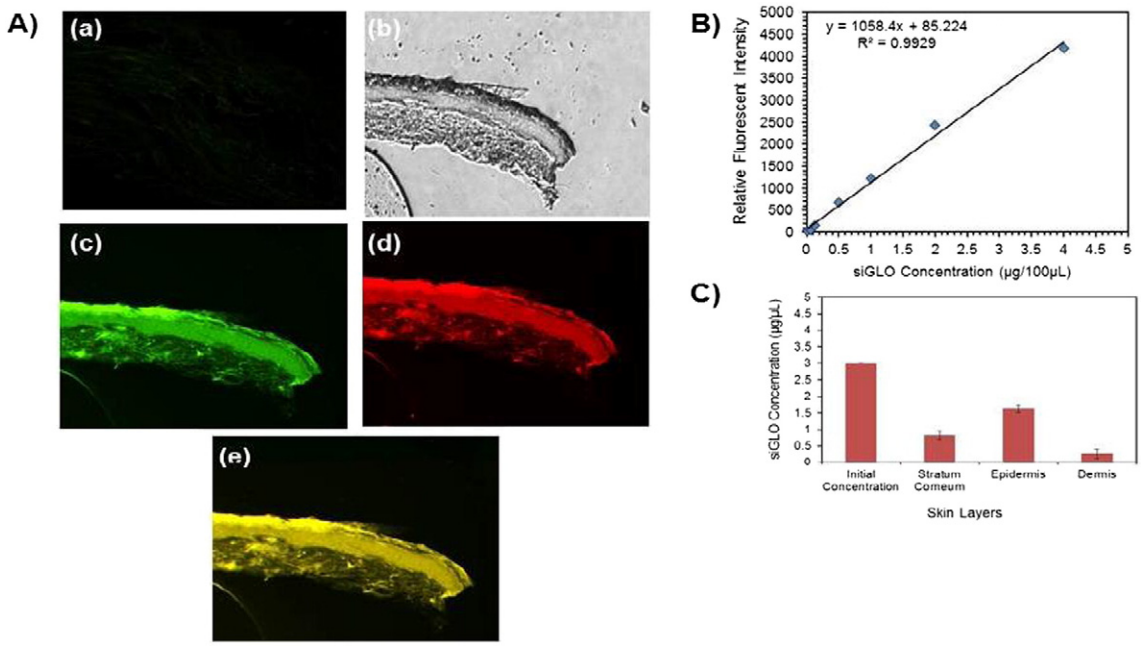

Fig. 5. A) Fluorescent microscope images of skin treated with lipoplexes prepared through complexation of green fluorescent labeled liposomes (liposome:NaChol ratio 8:1) and red fluorescent labeled siRNA (siGLO) (16:1 w:w). (a) This control panel shows the level of skin auto-fluorescence. (b) This panel shows the phase image of the skin defining stratum corneum, epidermis and dermis boundaries. (c) This panel shows permeation of green fluorescent liposomes in complex with siGLO through the skin layers. (d) This panel shows permeation of red fluorescent siGLO in complex with liposome that permeated through the skin layers. (e) Superimposition of panels c and d highlighting degree of colocalization. B) Calibration curve used for the quantification of siGLO in each skin layer. C) Quantification of siRNA in each skin layer based on the red fluorescence intensity of each layer. The initial concentration of siRNA was 3 μg. Data are reported as means ± s.d. (n = 3).

UACC-903 human melanoma cells were seeded at the density of 10,000 cells per well in 96-well plates and incubated at 37 °C for 24 h. Liposomes were then complexed with BRAF siRNA (0.5 μg) at the ratio of 16:1 (w:w) for 30 min and added to the wells (treatment group). Vehicle (HEPES buffer), empty liposomes, naked BRAF siRNA, liposomes complexed with scrambled siRNA were used as controls. After 24 h,

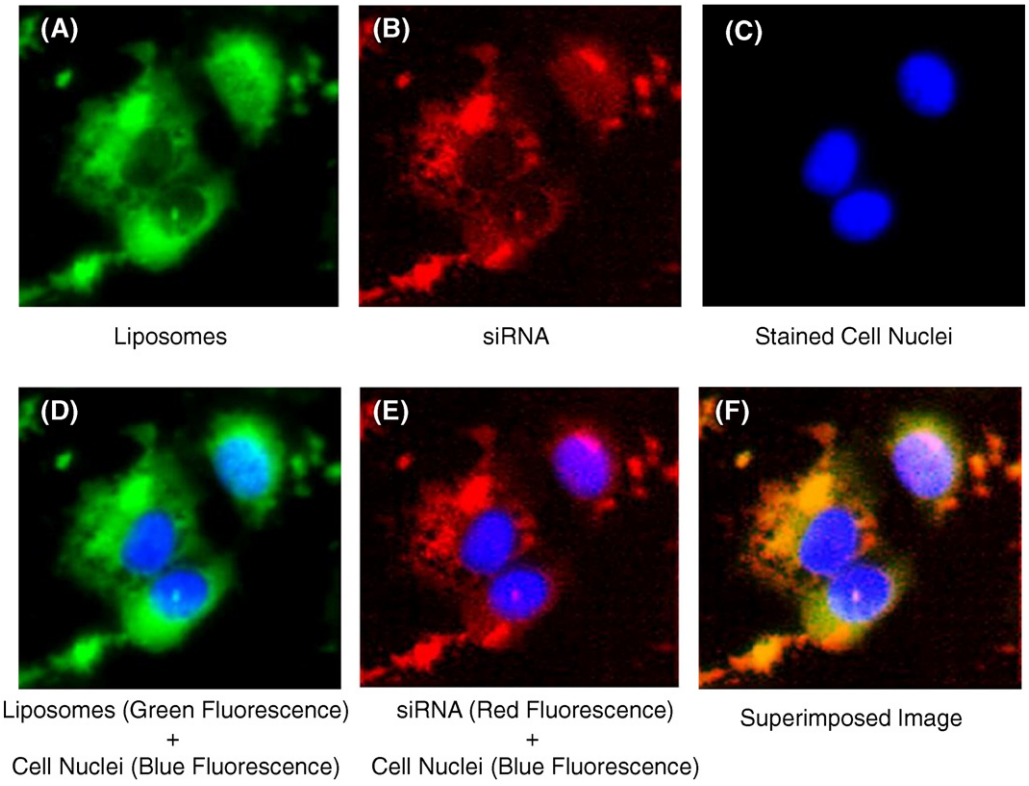

Fig. 6. Intracellular localization of liposomes and siRNA. Typical images of human UACC 903 melanoma cells incubated for 1 h with liposomes (green fluorescence) in complex with siGLO (red fluorescence). Cell nuclei were stained with nuclear-specific dye DAPI (blue fluorescence). Superimposed image shows co-localization of liposomes and siRNA.

cells were washed three times with DPBS and then fixed in formaldehyde 10% for 20 min. Cell membranes were permeabilized using a protein-free blocking buffer containing 0.1% Triton 100×. To block the non-specific sites, cells were incubated for 1 h in a protein free blocking buffer (Thermofisher Scientific Inc., NJ, USA). Cells were first incubated with anti-BRAF primary antibody and then with fluorescently labeled secondary antibody (PierceThermoScientific, IL, USA). After incubation for an appropriate time, cells' nuclei were stained with Hoechst 33258 (blue fluorescent dye) (Life Technologies, NY, USA) and visualized under a fluorescent microscope. The data were analyzed using ImageJ NIH software and reported as means ± s.d. (n = 3).

2.9. WST-1 cell toxicity assay

UACC-903 human melanoma cells were seeded in 96-well plates at the density of 10,000 cells per well and incubated at 37 °C for 24 h. Liposomes were mixed either with the control scrambled siRNA or BRAF siRNA at different concentrations equivalent to 0.1, 0.2, and 0.5 μg. After 30 min incubation at room temperature, cells were transfected with lipoplex formulations and incubated at 37 °C for 24 h. WST-1 reagent was added to each well and after 1 h incubation the absorbance of each sample was measured using a microplate reader at a wavelength of 450 nm. The data are reported as means ± s.d. (n = 3).

3. Results and discussion

3.1. Particles size, zeta potential and charge neutralization studies

Cationic liposomes were first prepared by using different w:w ratios of DOTAP as the cationic lipid and NaChol as an edge activator ranging from 4:1 to 10:1. The prepared liposomes were then characterized in terms of size and charge since both of these parameters have significant impact on nanoparticle permeation through the skin. The results of this study showed that as the DOTAP content increased, the size of the

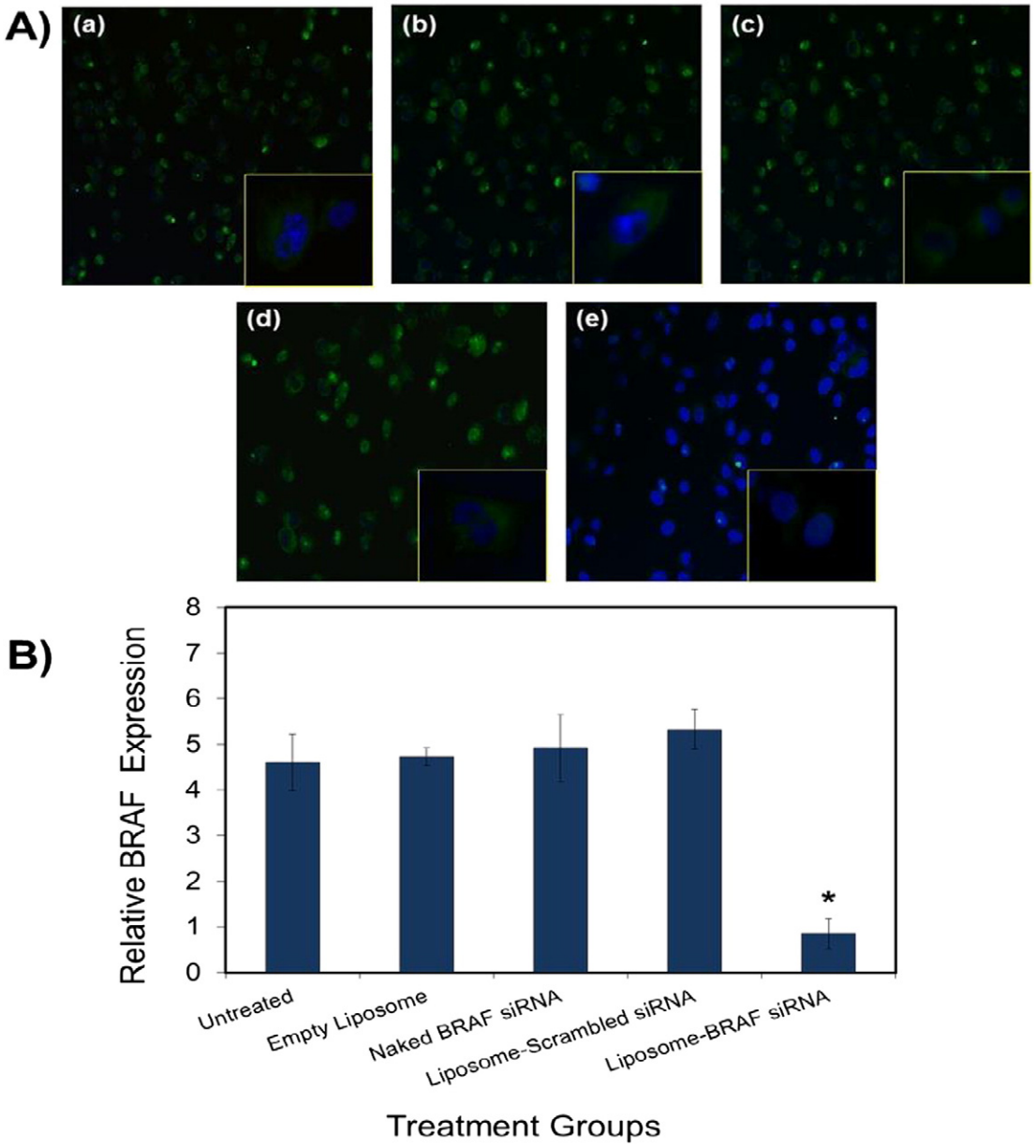

Fig. 7. In-cell immunofluorescence assay of UACC-903 melanoma cells treated with liposome/BRAF-siRNA. A) Qualitative analysis of BRAF protein expression knockdown in cells: (a) untreated; (b) treated with empty liposomes; (c) treated with naked BRAF siRNA; (d) treated with liposome containing scrambled siRNA; and (e) treated with liposome-BRAF siRNA complexes. B) Quantitative analysis of protein expression knockdown. Data are reported as mean ± s.d. (n = 3).

liposomes decreased from 120 nm to 60 nm and the surface charge increased from 25 to 37 mV (Fig. 1). The sizes of liposomes that were prepared with DOTAP:NaChol at ratios of 6:1, 8:1 and 10:1 were statistically in the same range from 60 to 65 nm (*t*-test, $p > 0.05$).

In the next step, the prepared liposomes at each DOTAP:NaChol ratio were complexed with siRNA to make lipoplexes ranging from 4:1 to 16:1 (liposome:siRNA). Formation of lipoplexes was based on the electrostatic interactions between positively charged liposomes and negatively charged siRNA. The lipoplexes were then characterized in terms of size and surface charge (Fig. 2).

The results of this study showed that all lipoplexes at 4:1 (liposome:siRNA) ratios had negative surface charges with sizes ranging from 120 to 200 nm. This suggests that at 4:1 ratio a portion of the siRNA molecules remained unneutralized indicating that the number of positively charged liposomes in the solution was not sufficient to complex with all siRNA molecules. To test this hypothesis, we performed a gel retardation assay on liposome-siRNA complexes. For this purpose, we chose DOTAP:NaChol liposomes at 8:1 ratio as an example and prepared liposome-siRNA complexes ranging from 1:1 to 16:1. Then, all lipoplexes were loaded onto an agarose gel followed by visualization of siRNA mobility retardation. The results of this assay confirmed our observations that lipoplexes at 1:1 and 1:4 ratios were not fully condensed as evidenced by the free unbound siRNA, whereas lipoplexes at 8:1 ratio or higher could fully neutralize siRNA charges and no free siRNA was detected (Fig. 3).

Further analysis of the particle size showed that as the liposome:siRNA ratio increased from 8:1 to 16:1, the sizes of the lipoplexes decreased in all formulations and stabilized to approximately 75 nm. Furthermore, in comparison to uncomplexed liposomes (Fig. 1), the surface charges of the lipoplexes at these ratios were reduced significantly from 37 mV to maximum 18 mV (Fig. 2).

Having learned that liposome-siRNA complexes at ratios higher than 4:1 are able to fully condense siRNA into small positively charged nanoparticles, we evaluated the ability of these formulations to permeate through the skin and reach the upper dermis. Since the liposome-siRNA complexes at 4:1 ratio had large sizes and negative surface charges, we used these in skin permeability studies as negative controls.

3.2. Evaluation of the skin permeability of liposome-siRNA complexes

To evaluate the ability of the formulated liposome-siRNA complexes (4:1 to 16:1 ratios) to diffuse through the human cadaver skin, DOTAP-NBD with green fluorescence was used to prepare the liposomes. Here, we used skin sections from one donor to make sure that the observed variations are due to formulation differences and not the donors. The qualitative and quantitative analysis of results showed that DOTAP:NaChol liposomes prepared at ratio of 8:1 and in complexation with siRNA at ratios of 8:1, 12:1 and 16:1 had the highest rates of skin permeation (Fig. 4A and B). Among these three formulations, liposomes prepared with DOTAP:NaChol at 8:1 ratio and complexed with siRNA at the 16:1 ratio had the highest rate of permeation through the skin layers with significant deposition at upper dermis. The analysis of green fluorescence in the skins treated with liposome-siRNA at 8:1 and 12:1 ratios showed diffusion of complexes into the lower layers of the dermis whereas lipoplexes at 16:1 ratio had significant deposition on the upper layers of dermis. Notable, that all three lipoplexes (8:1, 12:1 and 16:1 ratios) had almost identical sizes (~75 nm). Therefore, lipoplex size could not have contributed to such significant differences in skin permeation (Fig. 2). The fact that DOTAP:NaChol liposomes at 8:1 ratio (composed of 12.5% edge-activator) penetrated through the stratum corneum and deep into the epidermis more efficiently than other groups (i.e., 4:1, 6:1 and 10:1) could be attributed to the optimum balance of the lipid (DOTAP) and edge activator (NaChol) in the liposome structure. The balance of positively charged DOTAP to NaChol appears to have played a paramount role because at 8:1 ratio we could effectively penetrate the stratum corneum and reach the lower epidermis/upper dermis whereas at 10:1 ratio lipoplexes could overcome stratum corneum only and deposited at upper epidermis failing to reach lower epidermis. The fact that liposomes that were prepared at 10:1 DOTAP:NaChol ratio (composed of 10% edge activator) failed to reach the lower epidermis could be the result of insufficient edge activator in the liposome structure in order to propel them deeper into the skin. Interestingly, our results for DOTAP:NaChol at 6:1 ratio revealed that the lipoplexes could permeate through all skin layers and deposit uniformly throughout the dermis. This could be attributed to the higher percentage of edge activator in the liposome structure (i.e., 16.6%). In addition to the efficient permeation through the skin layers, it was interesting to observe that as the ratio of liposome (8:1) to siRNA increased, the deposition in the basal epidermis/upper dermis increased. It has previously been demonstrated that negatively charged liposomes equipped with edge activator and sizes of less than 100 nm can permeate through the skin layers and continue their journey until they reach the microvessels and enter the blood stream with minimal deposition into the skin [16]. Therefore, it is logical to hypothesize that positively charged lipoplexes which can easily interact with the negative charges on the surfaces of the cell membranes are prevented from further diffusion and remain in the basal epidermis due to internalization into the cells [17,18]. Overall, our findings highlight that particle size, surface charge, edge activator content and liposome:siRNA ratio play important roles in the effective permeation through stratum corneum/epidermis and deposition in upper dermis. These data impact the progressing in topical siRNA delivery since in the past it was thought that active delivery was necessary to transport siRNA in therapeutically significant levels to skin layers. In this study we have shown that passive delivery (dependent on a concentration gradient) is able to achieve similar levels of the active agent in the skin [2,3].

To investigate and quantify the amount of siRNA that was carried by the DOTAP:NaChol liposomes (8:1) into each skin layer, liposomes were complexed with the red fluorescently labeled siRNA (siGLO) at the 16:1 ratio and applied on the human cadaver skin. The analysis of data showed that lipoplexes could overcome the stratum corneum barrier, effectively permeate the skin and deliver the siRNA mainly to the basal epidermis and upper dermis (Fig. 5A-C). This observation also indicates that at this ratio liposomes remained in complex with siRNA and did not dissociate because it is well-understood that siRNA by itself and without the help of a carrier is unable to penetrate the stratum corneum [19]. We also analyzed the media in the Franz cell receptor compartment by fluorescence spectrophotometry to see whether any labeled liposome or siRNA could be detected. The results revealed that neither liposome nor siRNA was present in the media in detectible concentrations

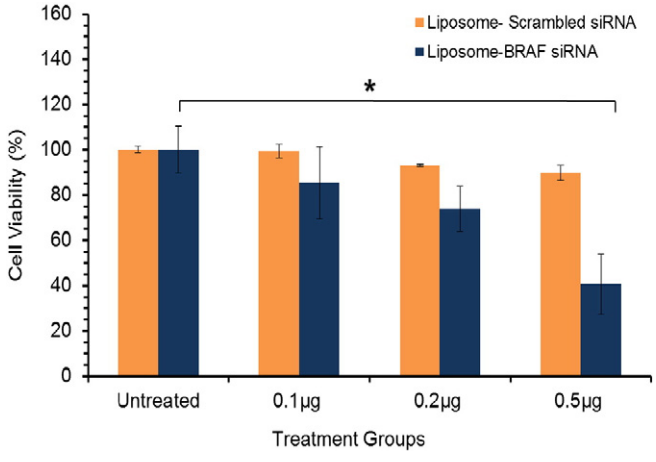

Fig. 8. WST-1 cell proliferation assay. Evaluation of toxicity in UACC-903 human melanoma cells treated with liposome-*BRAF* siRNA complexes (Equivalent of 0.1, 0.2 and 0.5 µg siRNA). Liposome-scrambled siRNA complexes at the same concentrations were used as controls. Data are reported as mean ± s.d. (n = 3).

suggesting that the lipoplexes did not cross the dermis to reach the receptor compartment. This is an important observation since it points at more localized therapeutic effects and less probability of observing systemic toxicity after administration.

3.3. Intracellular localization of the lipoplexes and protein expression knockdown

In the next part of the study we examined the ability of lipoplexes to internalize into human melanoma cells as they are the ultimate target for the developed siRNA delivery system. For this purpose, green fluorescent labeled DOTAP:Nachol liposomes were complexed with siGLO at different ratios and used to transfect UACC-903 melanoma cells. The results demonstrated that all lipoplexes could effectively internalize into melanoma cells, most likely due their positive surface charges. For example, Fig. 6 shows the ability of the DOTAP:Nachol liposomes (8:1) in complex with siGLO at 16:1 ratio to internalize and localize in the cytoplasm of UACC-903 melanoma cells; thereby, raising the possibility of effective protein expression knockdown in these cells. This particular formulation of lipoplexes was chosen based on the previous experiments that showed the highest rate of permeation through the skin layers.

To examine whether the lipoplexes could release the siRNA inside the cytoplasm which in turn would result in protein expression knockdown, we performed the next set of studies using therapeutic BRAF siRNA.

3.4. Evaluation of BRAF expression knockdown by lipoplexes

In the next step, we evaluated the ability of the liposomes in complex with BRAF (*V600E*) siRNA to knockdown the protein expression. BRAF is a serine/threonine kinase protein involved in the MAPK/ERK signaling pathway and upon activation, it signals for the cell process regulation such as gene expression, cell proliferation, differentiation, and apoptosis [20]. In the case of an oncogenic gene mutation in the MAPK pathway, cells grow and differentiate aggressively and develop tumors. As was mentioned previously, 60% of melanoma cases are caused by a point mutation in the gene that encodes BRAF protein and 90% of these mutations involve substitution of valine for glutamic acid at the 600 amino acid of the protein (V600E). Therefore, by delivering siRNA targeted to the mutated *BRAF* RNA, the expression of the BRAF protein can be attenuated which can consequently reduce melanoma cell proliferation.

Using *BRAF* (*V600E*) siRNA, lipoplexes at 16:1 ratio were prepared as mentioned above and used to transfect UACC-903 melanoma cells. This combination of siRNA and melanoma cell line was chosen since the selective silencing effect of the *BRAF* (*V600E*) siRNA on UACC-903 and other human melanoma cells has been reported in the literature [21–24]. The BRAF protein expression levels inside the treated cells were measured by using in-cell immunofluorescence assay (Fig. 7). The results of this study did not reveal BRAF protein expression knockdown in cells that were treated with empty liposomes, naked BRAF siRNA or liposomes in complex with scrambled siRNA (control groups). In contrast, the BRAF protein expression level was significantly reduced in cells that were treated with liposomes in complex with BRAF siRNA. This indicates that the lipoplex formulation could internalize, release the therapeutic siRNA into the cytoplasm of melanoma cells and effectively down regulate the expression of the BRAF protein.

We further examined the ability of the formulated lipoplexes carrying BRAF siRNA to inhibit the proliferation of melanoma cells. Therefore, lipoplexes were prepared at 16:1 ratio using BRAF-siRNA (ranging from 0.1 to 0.5 μg) and then used to transfect UACC-903 melanoma cells. The impact of the prepared lipoplexes on melanoma cell proliferation was evaluated by using WST-1 cell proliferation assay. UACC-903 human melanoma cell line was used as a model because it has been reported that this cell line contains high levels of mutated *BRAF* V600E [23,25]. The results of this study showed that lipoplexes carrying at least 0.5 μg of BRAF siRNA (equivalent to 120 nM) were able to significantly reduce the viability of the cultured melanoma cells (Fig. 8). In contrast, liposomes carrying 0.5 μg of the scrambled siRNA did not induce any toxic effects on melanoma cells. Overall, it appears that our developed liposomal system is efficient in BRAF knockdown since it requires 0.5 μg siRNA (120 nM). This is significantly more efficient than what is reported in literature with other liposomal delivery systems which require BRAF-siRNA in 1 μM range [26]. Therefore, the optimum formulation identified in this study seems to be efficient not only in permeating the skin layers but also gene knockdown.

In parallel to liposomal systems, other siRNA delivery systems are also under development. For example, Hsu et al. (2011) have reported the use of a peptide-based delivery system, namely SPACE that can permeate through the skin layers and deposit macromolecules in lower epidermis [27]. Later, Desai et al. (2013), reported a lipid/polymer hybrid nanosystem that could deliver siRNA to lower epidermis [28]. Although the above mentioned siRNA delivery systems have not been studied side-by-side to determine which is the most efficient one, but the fact that more than one system is available for topical delivery of siRNA to lower epidermis significantly increases the rate of success in in vivo studies for melanoma therapy.

4. Conclusions and future studies

The results of this study demonstrate that for the efficient siRNA delivery through the skin layers (i.e., stratum corneum and epidermis) and deposition in the upper dermis a correct balance of lipoplex size, charge and edge activator content is required. The developed lipoplexes were able to not only permeate through the skin layers but also effectively internalize into the viable cells of basal epidermis and knockdown the expression of target proteins. Given that no active delivery approach such as ultrasound was used to enhance skin permeation, the developed liposomal system can be considered a major step forward towards a simple and efficient drug delivery of macromolecules via the topical route for therapy of various skin diseases. In near future, it is our intention to develop a lipoplex-embedded hydrogel system to enhance local retention time of the lipoplexes on the skin and make a suitable formulation for in vivo studies. It is also noteworthy that in this study we used skin from one donor to ensure that the observed differences are due to formulation variables and not the donors. To demonstrate the broad application of the developed formulation, in future the efficacy of the system needs to be tested in different skin types considering race, age and gender.

Conflict of interest

The authors claim no conflict of interest.

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