Targeted miRNA delivery for psoriasis treatment based on modified starch and ultrasound application

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ABSTRACT SUMMARY
Psoriasis is a skin disease that affects millions, and still without a cure. miRNAs are promising molecules for psoriasis gene therapy\textsuperscript{1}, but delivering it to specific cells entails problems in penetrating the skin, avoiding enzymatic degradation and eventually, penetrating to the basal keratinocytes (KC) cells. Our \textit{in vitro} and preliminary \textit{in vivo} results verified the ability of ultrasound (US) and modified starch carrier to enhance miRNA/siRNA transdermal delivery, as well as cell entrance.

INTRODUCTION
Psoriasis is a chronic inflammatory skin disease caused by rapid and incomplete differentiation of skin basal cells, building up thick and red skin patches with flaky, silver-white patches called scales. The psoriasis treatments that are offered today are topical treatments, phototherapy and systemic medications based on regulation of KC or controlling the immune system. In spite of a range of options, effective treatment of psoriasis can be challenging, since the disease is unpredictable and the skin can become resistant to various treatments over time. Moreover, the most potent psoriasis treatments can have serious side effects.

RNA interference (RNAi) is a natural process of sequence-specific post transcriptional gene silencing to inhibit gene expression. One of the miRNAs which differs between normal and psoriatic involving skin is miR-197\textsuperscript{1}, which can be an attractive therapeutic molecule for psoriasis treatment since it is down regulated in psoriatic lesions and can affect the differentiation, proliferation and migration of KC (preliminary results obtained in Y. Sidi's Lab)

Two major barriers to topically delivered RNAi therapy exist: 1. The barrier properties of the top layer of the epidermis (stratum corneum) 2. Naked miRNA/siRNA delivery is unstable \textit{in vivo} due to enzymatic degradation and immunological responses. Moreover, the efficiency of RNAi that does reach the target cells is further limited by poor cellular uptake.

To overcome these obstacles and allow topical delivery of miRNA to skin cells, one must consider the use of designed carrier for its safe and efficient delivery to the desired target cells, combined with methods that enhance transdermal delivery. In this study, we suggested the use of US as a mean to enhance biological membranes and skin permeability\textsuperscript{2-3} and modified starch as an miRNA delivery carrier. By combining these two approaches, the ability to bypass limiting barriers of skin permeability and KC cell transfection path is of high potential. This might significantly improve current treatment and also pave the way for future miRNA-based therapy.

EXPERIMENTAL METHODS
Carrier synthesis and complexes formation: starch modification with quaternary amine groups (3-Chloro-2-hydroxypropyltrimethylammonium chloride- reagent) to obtain quaternized starch (Q-starch) was carried out according to Geresh \textit{et al}\textsuperscript{4}. Complexes of Q-starch and siRNA/miRNA were prepared at the optimal N/P molar ratio of 2 (molar ratio between positive nitrogen groups on Q-starch (N) and negative phosphate groups on siRNA backbone (P)). Q-Starch solution (in distilled water) was introduced into a tube containing RNAi solution. The tube was incubated for 40min until complexation was established.

Cellular uptake: \textit{In vitro} experiments were performed on Keratinocyte HaCaT cells seeded in a 12-well plate with a glass coverslip, 24h before transfection at a density of 6*10\textsuperscript{5} cells/well, reached 40-50% confluence on the day of experiment. On the day of transfection, the culture medium was removed and 900µL of serum plus antibiotic free media was added to each well. Different concentrations (5nM to 1µM) of labeled Q-starch/siRNA complexes were evaluated. As control, naked siRNA was used.

In order to evaluate the US contribution to the cellular uptake, a 12-well tissue culture plate was placed in aqueous medium above 20KHz US (Qsonica Sonicator Q700 System) plate horn bath. US was applied for 20 sec at 2.14 mW/cm\textsuperscript{2}, 60min after complexes were introduced to the cells. Complex incubated for additional 3h. After complexes' incubation, the cells were fixed with 4% paraformaldehyde and the membrane labeled with
fluorescent dye. Complexes uptake was determined by confocal microscopy.

**Human skin penetration:** *In vivo* experiments were evaluated on SCID mice transplanted with human skin (Helsinki permit number 815) (engrafted SCID mice, Israeli health ministry application number 5528). US medium and labeled Q-starch/miRNA complexes were placed inside a small chamber (13mm diameter) that was located on the transplanted human skin. US medium was replaced every 30sec, in order to eliminate temperature increase. To evaluate the permeability of the skin, conductivity measurements were conducted during ultrasound exposure. The US (8.2 W/cm²) was turned off when conductivity reached 40-60 fold of the initial conductivity. 18h after the termination of the experiment the mice were sacrificed. Complexes uptake was determined by confocal microscopy of histological samples of the skin.

**RESULTS AND DISCUSSION**

Confocal results presented in Figure 1a and 1b demonstrate the ability of Q-starch/siRNA complexes (0.5µM and 1µM, respectively) to enter human KC HaCaT cells while low concentration complexes (0.05µM) and naked labeled siRNA did not enter these cells as can be seen in Figure 1c and 1d, respectively.

**Figure 1:** Confocal images of KC HaCaT cells incubated for 4.5h with Q-starch/siRNA<sub>55</sub> complexes (N/P 2) at different concentrations: a. 1µM, b. 0.5µM, c. 0.05µM, and d. Naked siRNA<sub>55</sub>concentration was 1µM. Cells' membrane was labeled with WGA in red, and siRNA<sub>55</sub> was labeled in blue.

US affect cell membrane permeability causing increased cellular internalization, and hence, can increase the efficiency of the delivery mechanism. Our results confirmed the beneficial effect of US application as an added means to reach higher efficiency of complexes' entrance to HaCaT cell line, as can be seen in confocal images presented in Figure 2. Moreover, US application allows to introducing the cells siRNA concentration at two orders of magnitude lower then what was needed for transfection without ultrasound (Figure 1).

**Figure 2:** Confocal images of KC HaCaT cells incubated for 4h with Q-starch/siRNA<sub>55</sub> complexes at N/P 2. Total siRNA concentration in the medium was 0.05µM, where: a. with US application, and b. without US application. Cell's membrane labeled with WGA in red, and was siRNA<sub>55</sub> labeled in blue.

Preliminary *in vivo* experiments on SCID engrafted mice verified the ability of US application that enables complexes' entrance through the stratum corneum to the basal layer. As can be seen in Figure 3, Complexes can be seen at the basal KC cell layer of the epidermis only after treatment with US.

**Figure 3:** Skin cross section: penetration of Cy5-miR-197 through transplanted human skin on SCID mice: a. following the sonication a solution of Q-Starch/Cy5-miR<sub>197</sub> complexes (N/P 2,4µM) was placed on the exposed skin, b. the Q-Starch/Cy5-miR-197 complexes were placed on the human skin without sonication.

**CONCLUSION**

The use of US combined with Q-starch complexes has a potential to be a efficient technology that allows RNAi to be delivered across the stratum corneum and allow uptake by keratinocytes.

**REFERENCES**