Topical Sensory Nanoparticles for *in vivo* Biomarker Detection

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ABSTRACT

We have recently developed a non-toxic, topically applied biosensor using solid lipid nanoparticles (SLNs). SLNs are similar to nanoemulsions except that the oil phase is held below its melting point. The hydrophobic core functions as a host for the delivery of a wide variety of lipophilic substances and the hydrophilic surfactant corona provides a functionalization platform for a wide range of chemically, biologically, and optically active molecules. Our diagnostic platform is currently designed to continuously monitor hydrogen peroxide levels in skin. Overproduction of hydrogen peroxide is implicated in the development of many diseases, and may indicate early infection as well as exposure to toxins or radiation.

Keywords: nanoparticle, sensor, peroxide, disease

1 INTRODUCTION

The utilization of biocompatible, sensory nanomaterials has great potential for pain-free diagnostic strategies. Nanomaterials can be designed for compatibility with human tissues and are amenable to loading with pharmaceuticals or sensory compounds. The skin is a prominent target organ for numerous inflammatory and stress-related signals that alter during disease, and it offers a rich environment for biomarker exploration [1].

The skin is an excellent barrier and prevents the ingress of chemical and biological agents due to its complex structure and composition [2]. Despite these properties, the skin is a desirable site for topical delivery of pharmaceuticals for sensing or treating disease. Skin cells produce reactive oxygen species in response to a variety of insults including chemicals, pathogens, and ultraviolet radiation. Overproduction of hydrogen peroxide has been specifically associated with skin disease (e.g. cancer) and the progression of normal wound healing processes [3].

Solid lipid nanoparticle (SLN) formulations are particularly amendable to biomedical skin applications. SLNs have been shown to penetrate human and pig skin more rapidly and to a greater extent than conventional vehicles. This enhanced penetration is thought to arise from the fact that SLNs form an occlusive layer on the skin surface, following the evaporation of water from the topically applied lipid nanodispersion. The resulting increased hydration of the stratum corneum then facilitates intercellular penetration [4].

We have developed a SLN formulation that can detect small quantities of hydrogen peroxide. The chemical interaction of the particles with hydrogen peroxide results in excitation of the incorporated fluorescent dye. This reaction can be tailored to a specific application by altering the incorporated dye, and is easily measured by standard optical detectors. We have characterized these particles and their compatibility with skin, in addition to exploring their limit of detection for the biomarker, hydrogen peroxide.

2 EXPERIMENTAL

2.1. Materials

Eicosane (C20, Lipid) (Aldrich), Brij 97 (C18E10) (Aldrich), 9,10-is(phenylethynyl)-antracene (TCI, Dye), 1-1'-Oxalyldiimidazole (TCI), Methyl Alcohol (Sigma-Aldrich), Decyl Alcohol (Sigma), and DI Water.

2.2. Nanoemulsion Preparation Procedure

SLNs were prepared by the phase inversion temperature (PIT) method [5-7]. In this method, the composition remains constant while the temperature is changed. 9,10-bis(phenylethynyl)-antracene, 1-1'-oxalyldiimidazole, methyl alcohol, and decyl alcohol were combined into a vial. The following step was the addition of Brij 97 and eicosane. The resulting mixture was co-melted at 60 ˚C and stirred. DI water was added to the mixture, heated and stirred. Under continued stirring, cooling the sample causes inversion of the water-in-oil emulsion to an oil-in-water emulsion, creating a nanoemulsion in the process. A further decrease in temperature below the melting point of the lipid produces SLNs from the emulsified droplets.

2.3. Analyses

2.3.1. SLN Characterization

Particle size analysis was performed using Dynamic Light Scattering (DLS) which uses frequency shifted light to

measure nano-size particles. A Mettler-Toledo Differential Scanning Calorimeter DSC1 with Star software was used to study the thermal stability of the nanoparticles.

2.3.2. MTT assay

Toxicity testing. (Cell culture) Human keratinocytes (HaCaT, Cell Lines Service) were cultured in DMEM, with glucose and L-glutamine, without sodium pyruvate (Mediatech), 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cells were maintained in a humidified atmosphere at 37˚C, 5% CO_2 and passaged at 80% confluence. (MTT assay) HaCaT cells were plated at a density of 4e4 cells/well and allowed to equilibrate at 37°C overnight. 5µl/well of the nanoemulsion was added to each replicate well. After 18 hours of exposure to the nanoparticle preparations, cellular health was determined using the MTT assay, according to the manufacturer's protocol (ATCC). Briefly, MTT reagent was added to the wells of the microplate and after two hours of incubation at 37°C, intracellular formazin crystals were solubilized with the provided detergent solution. Absorbance values were obtained using the Safire2 microplate reader (Tecan US, Raleigh, NC) with a measurement wavelength of 570 nm and a reference wavelength of 700 nm, read from the bottom.

2.3.3. Hydrogen Peroxide Detection

Nanoparticles (100 µl) were pipetted into the wells of a white, flat-bottom, 96-well microtiter plate. Hydrogen peroxide was diluted in distilled water and 5 µl was added to each appropriate particle sample. Sample chemiluminescence was immediately read using the Safire2 microplate reader (Tecan US, Raleigh, NC) and was measured every two minutes for sixty minutes. Values for duplicate wells were averaged and the standard deviation determined.

2.3.4. Penetration of Porcine Skin

Fresh, intact porcine ear skin was cleaned with 70% ethanol and rinsed in distilled water prior to experimentation. The skin was separated from the cartilage and a small square was cut to fit in the static Franz diffusion cell. Skin was mounted in the cell and 100 µl of each sample was applied to the surface. Diffusion cells were sealed with parafilm according to the manufacturer's instructions (PermaGear) and incubated at room temperature, stirring, for 24 hrs. After incubation, residual samples were decanted and the surface of the skin was washed three times, using 500 µl sterile 1X PBS. A sterile, Dacron swab was used to remove the remaining wash solution and a 5 mm punch biopsy was taken from the center of the skin. Biopsies were immediately frozen in CryO-Z-T compound (Pelco) for cryosectioning. Cryosectioning of skin biopsies was performed by AML Laboratories (Baltimore, MD). Images of cross-sections were taken using a fluorescence microscope (Zeiss).

3 RESULTS AND DISCUSSION

3.1. SLN Characterization

We initially measured the physical characteristics of the SLN formulation by two methods. Both dynamic light scattering and differential scanning calorimetry were used to measure the particle size and thermal stability, respectively. As shown in figure 1, DLS analysis showed an average particle diameter of 22.4 nm, with low polydispersity. The SLNs in this size range provides the potential for deeper penetration in the skin in comparison with micron-sized formulations.

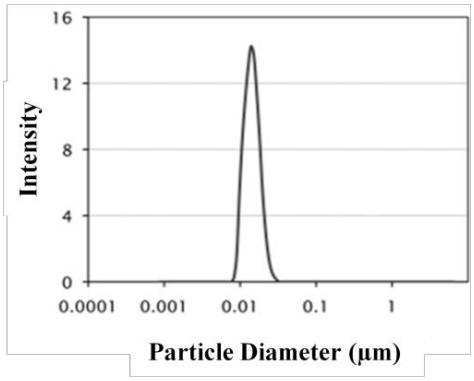

Figure 1: Particle size distribution of a nanoemulsion

The thermal stability of the nanoparticles was studied using DSC. The results showed a melting temperature of 32.7 ˚C for SLN using C20 as a lipid, which is ideal for skin application. We also explored the utilization of a variety of lipids, which resulted in a variation in the melting temperature of the SLN (data not shown). The melting temperature for SLN using nonadecane (C19) and triacontane (C30) as a lipid is 27.7 and 61.8 ˚C, respectively. The variation in the number of carbon (19 to 30) in the lipid gives a 34.2 ˚C window range in the melting of SLNs, giving total control over the melting temperature of the nanoparticles.

3.2. MTT assay

As our intended application for the peroxide-sensing particles involves topical administration to human skin, we next sought to determine the toxicity of the particle

formulation by several methods. The MTT assay is a standard *in vitro* viability assay that measures the effects of a chemical agent on cellular health (figure 2). We initially tested the toxicity of the nanoparticle formulation using HaCaT immortalized keratinocytes as a model (figure 2A). The viability of cells treated with nanoparticles was not significantly different than untreated cells or those treated with a low percentage of surfactant. In addition, we utilized a three-dimensional human skin equivalent model to estimate the *in vivo* irritancy potential of our formulation (figure 2B). Using the skin equivalents we were able to determine an approximate ET50 (effective time- 50) of 15hrs, which indicates that our formulation's expected *in vivo* irritancy is "very mild." According to the product manufacturer standards (see MatTek Corporation. "MTT Effective Time-50 Protocol".)

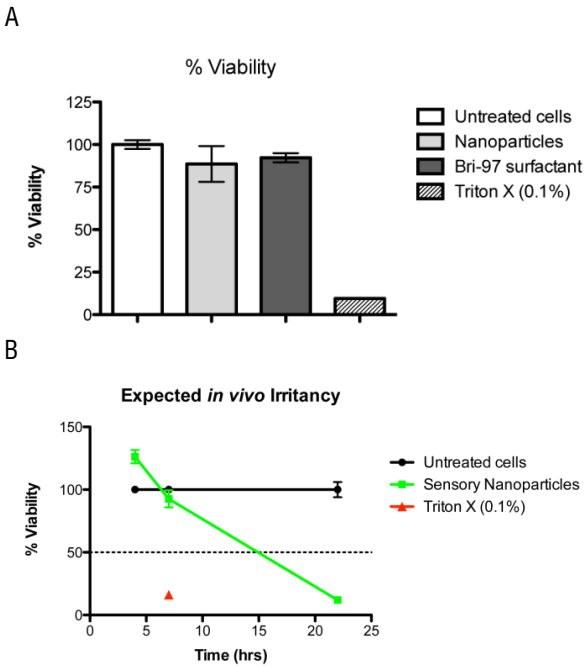

Figure 2: MTT toxicity testing

3.3. Hydrogen Peroxide Detection

In order to explore the applicability of this nanosensor to detection of hydrogen peroxide *in vivo,* we next wanted to determine the limit of detection of our formulation. Using a small volume of nanoparticles, we added exogenous hydrogen peroxide and measured the resulting chemiluminescence over the course of one hour. The data shown represent duplicate samples after addition of exogenous dilutions of hydrogen peroxide, using a lotion only sample as the baseline. Error bars represent the standard deviation of the mean.

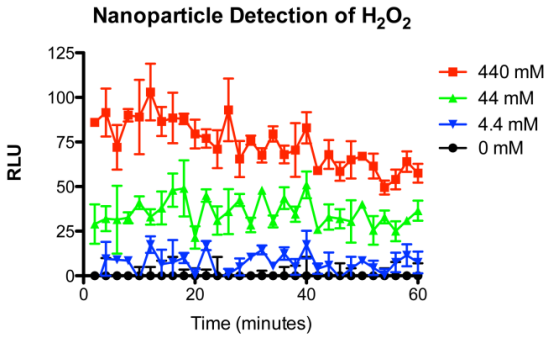

Figure 3: Detection of Exogenous Hydrogen Peroxide

As hydrogen peroxide is only produced by living cells within the skin, we were interested in determining to what extent the particles were able to penetrate the dead, outer layers of the stratum corneum. We applied the particles to fresh porcine skin mounted in standard Franz diffusion chambers. These samples were incubated overnight prior to washing, punch biopsy, cryosectioning, and fluorescence imaging (figure 4). The upper and lower, left-hand portion of the figure shows white light images and the upper and lower right-hand portion of the figure shows the corresponding fluorescence images. The upper images in this figure show attachment and penetration of 20 nm polystyrene beads, while the lower two images demonstrate the attachment and penetration of our nanoparticle formulation.

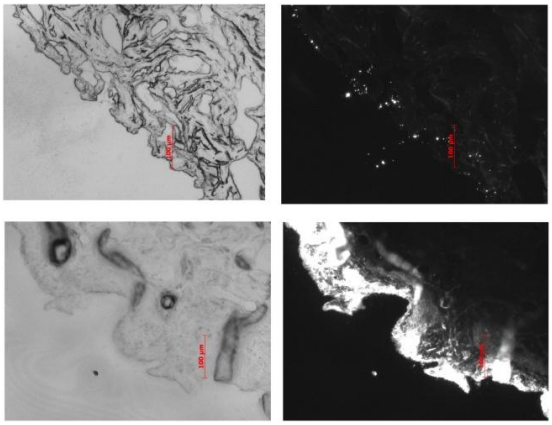

Figure 4: Nanoparticle penetration of porcine skin

4 CONCLUSIONS

We determined that the SLN formulation described in this study is well-suited to topical biosensing applications. We found the PIT method to be a versatile fabrication method where the size and thermal stability of the solid lipid nanoparticles can be easily tailored. The ability of the method to synthesize SLNs approximately 20 nm in size is expected to allow deeper skin penetration than larger particle

formulations. In addition, the variation in the melting temperature provides thermal stability depending on the environmental condition and/or application.

After examining the toxicity of our particles by two methods, we determined that the particles did not significantly affect cell viability and are not expected to cause irritation *in vivo*. These conclusions were verified in an *in vivo* hairless mouse model, where compounds were applied directly to mouse skin. No skin irritation or gross changes in animal weights were observed.

From these experiments we were able to measure micromolar amounts of exogenously added hydrogen peroxide with respect to background controls. The concentration of hydrogen peroxide in the skin during various disease conditions is unknown. However, to improve sensitivity, the limit of detection of the SLNs could potentially be improved by using a higher concentration of applied nanoparticles, or by altering the concentration of the oxalyldiimidazole within the particles. In future studies, we would like to use these particles to measure hydrogen peroxide from metabolically active cells. This will provide valuable information as to what physiologically relevant peroxide levels can be expected from a specific insult (e.g. chemical, ultra-violet radiation, etc.). In addition, this would help to establish the limits of detection using these particles and their expected activity over time.

Considering that the stratum corneum of pig ear skin is approximately 20 μm (8), and we estimate that our particles are penetrating beyond this layer, to areas of viable epidermis. Therefore cellular sources of hydrogen peroxide would be expected to come into contact with the particles, allowing topical detection of luminescence.

In future work, we envision further developing our SLNs as a platform for pain-free, topical diagnostics, tailored to the detection of a variety of specific biomarkers found in skin, including hormones, DNA, cytokines, and cholesterol. Pain-free clinical methods to assess these biomarkers could provide a valuable tool for better understanding a variety of disease states and environmental exposures.

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