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5-Aminolevulinic acid coated microneedles for photodynamic therapy of skin tumors

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ABSTRACT

This study evaluated the potential of coated microneedles for improved dermal delivery of 5-aminolevulinic acid (5-ALA), which naturally gets converted by cells of the tissue in to a photosensitizer called protoporphyrin IX (PPIX). Microneedle patches containing 57 microneedles were coated with 5-ALA using an in-house developed micro-precision dip coater. The coating process was optimized to achieve higher 5-ALA loading on microneedles and a high delivery efficiency into porcine cadaver skin. Using 5 dips with 25% *w/v* 5-ALA solution, a mass of about 350 μg of 5-ALA was coated per patch, which gave a delivery efficiency of about 90% in porcine cadaver skin. Bright-field and scanning electron microscopy established that coatings of 5-ALA on microneedles of the patch were uniform. *In vivo* dermal pharmacokinetics showed that delivery of just 350 μg of 5-ALA using coated microneedles led to about 3.2-fold higher PPIX formation after 4 h, as compared to topical application of 20% *w*/w 5-ALA in a conventional cream formulation (25 mg cream). Furthermore, with use of coated microneedles, PPIX was observed in deeper regions of the skin (~480 μm) as compared to topical 5-ALA cream formulation (~150 μm). The potential of PPIX for photodynamic therapy was tested *in vivo*. After light exposure (633 nm; 118 J/cm^2), PPIX got photosensitized, and due to higher initial amount of PPIX in the coated microneedle group, about twice the amount of PPIX was photobleached compared to topical cream application. Finally, even with a lower dose of just 1.75 mg 5-ALA, coated microneedles suppressed the growth of subcutaneous tumors by ~57%, while a topical cream containing 5 mg of 5-ALA did not suppress the tumor volume and led to tumor growth comparable to the untreated control group. Overall, the strategy of delivering 5-ALA using coated microneedles could be a promising approach for photodynamic therapy of skin tumors.

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1. Introduction

In recent years, photodynamic therapy based on 5-Aminolevulinic acid (5-ALA) has been widely employed for the treatment of different forms of skin neoplasms such as actinic keratosis, basal cell carcinoma, squamous cells carcinoma and cutaneous T-cell lymphoma [1,2]. 5-ALA by itself is not a photosensitizer (PS), however, it is a biological precursor of protoporphyrin IX (PPIX), which is a potent PS. As part of the natural cell physiology, 5-ALA is synthesized inside cells, and transformed into PPIX in a multi-step reaction inside the mitochondria, where it produces heme. Free heme can regulate endogenous production of 5-ALA through a negative feedback mechanism [3]. Exogenous administration of 5-ALA in high doses usually bypasses this feed-back control process, which then leads to intracellular accumulation of PPIX, presumably because excess PPIX produced cannot be efficiently converted to heme. In clinical practice, 5-ALA based photodynamic therapy is facilitated by delivering a high dose of 5-ALA into the skin lesions or nodular tumors, and then exposing the site with light at a fixed wavelength to activate PPIX that is formed *in-situ* from 5-ALA. PPIX activation produces reactive oxygen species (ROS) in the presence of molecular oxygen and leads to apoptosis and necrosis of cancer cells [4,5]. Commercially, 5-ALA is available as a topical solution (20% Levulan®, DUSA Pharmaceuticals Inc.) and is approved for the treatment of hypertrophic actinic keratosis on the face and scalp in combination with blue light illumination (BLU-U®) [1]. Other formulations of 5-ALA such as a topical patch (Alacare®, Spirig Pharma AG, Switzerland) and a gel (Ameluz®, Spirit Healthcare Ltd., Oadby, UK) are also approved in combination with red light [1]. The key benefits of 5-ALA-based photodynamic therapy include tumor selective localization and rapid elimination of PPIX from the body (shorter half-life), which leads to reduction of skin photosensitization by natural light [6].

Despite these advantages, a major limitation of 5-ALA-based treatment is the poor penetrability of 5-ALA through the lipidic barrier of the skin (stratum corneum) due to high polar characteristics (Log P; −1.5) and zwitterionic nature of 5-ALA. This overall leads to poor efficacy of 5-ALA in photodynamic therapy of superficial skin lesions, specifically in nodular tumors, where a higher concentration of drug is usually required at a depth of 2 or 3 mm from the surface of the skin lesion [7]. To increase penetration of 5-ALA into the stratum corneum,

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curettage or debulking of nodular lesions is often performed, which is usually associated with excessive pain and discomfort to the patients. A variety of novel delivery approaches such as lipophilic pro-drugs [8–10], penetration enhancers [11], thermo-gelling formulations [12], PLGA nanoparticles [13], ethosomes [14] and nanoemulsions [15] have been investigated to increase dermal penetration of 5-ALA. However, efficient utilization of these approaches is limited due to unfavorable skin toxicity or sensitivity, low drug loading, and poor chemical stability of 5-ALA.

Microneedle-technology offers a distinctive method of cutaneous drug delivery. Using microneedles, drug can be delivered through the stratum corneum with drug-coated microneedles, drug-encapsulating microneedles, hollow microneedles, or application of the drug on skin punctured with microneedles [16]. The precisely controlled dimensions of microneedles in the micron-size range cause minimal pain at the application site [17], and the potential of self-administration for some applications can considerably increase the patients' compliance [18]. Dermal penetration of 5-ALA has previously been improved using the microneedle based poke-and-patch approach, where the skin surface was punctured with microneedles to create micro-pores before the application of a drug-containing patch or formulation [19–21]. However, the poke-and-patch approach is limited by quick recovery of skin, which leads to rapid resealing of pores generated by the microneedle pre-treatment step [22–25].

In recent years, coated microneedles have been used to deliver a broad spectrum of drugs and biomolecules [26–37] in to the skin, including water insoluble drugs using molten coatings [38]. Leveraging our previous experience on microneedle coating technology, we investigated, for the first time, the potential of a coated microneedle patch (containing 57 microneedles) to increase cutaneous delivery of 5-ALA. In the present study, we first optimized critical coating parameters to maximize both the mass of 5-ALA coated on to microneedles and its delivery efficiency in to porcine skin *in vitro*. We then evaluated the ability of 5-ALA coated microneedles to generate PPIX in the skin of Balb/C mice *in vivo*, and compared it with topical application of 20% 5-ALA (mixed with inactive cream base) on top of intact skin or skin punctured with uncoated microneedles. The depth and localization of PPIX in mice skin, and the photo bleaching response of PPIX by red light (633 nm) was also determined. Finally, the efficacy of 5-ALA coated microneedles for photodynamic therapy was tested in a mouse tumor model using A-20 cells (mouse B lymphoma cells).

2. Materials and methods

2.1. Materials

5-Aminolevulinic acid, fluorescamine, protoporphyrin IX base, phosphate-citrate buffer (pH 5.5) and borate buffer (pH 8.5) were purchased from Sigma-Aldrich (MO, USA). Carboxymethylcellulose sodium salt (low viscosity, USP grade) was obtained from CarboMer, (CA, USA). Lutrol F-68 NF was procured from BASF (NJ, USA). Inactive cream base (Unguentum M®) was purchased from Almirall Hermal GmbH (Germany). 2-Mercaptoethanol, Penicillin-Streptomycin (10,000 U/ml) and fetal bovine serum were purchased from Gibco® (PA, USA). RPMI-1640 medium was procured from American Type Culture Collection (ATCC) (VA, USA). Dimethyl sulfoxide (DMSO, AR grade), acetonitrile (HPLC grade), methanol (HPLC grade) and crystal violet were purchased from Fisher Scientific (PA, USA). Ultra-pure deionized water (Millipore, Direct Q® 3 UV) was used for all the experiments. All other reagents used were of analytical grade.

2.2. Microneedle fabrication and dip-coating process

Two dimensional (2D) microneedle arrays containing 57 microneedles were fabricated from stainless steel (304) sheets (thickness; 50 μm) *via* wet etch process [37]. Subsequently, manual bending of individual microneedles was carried out to make them perpendicular to the sheets. Each microneedle in the 2D patch was 700 μm long and 200 μm wide.

Microneedle patches were coated using a micro-precision dip coating station developed in house. Microneedles were mounted on a coating stage which comprised of automated x-y computer controlled linear motorized stages. The coating solution was filled in a micro-reservoir fitted on one of the stage. Each microneedle of the patch was coated by dipping into the micro-reservoir. The coating solution consisted of 1% (*w*/*v*) carboxymethylcellulose sodium salt, 0.5% (*w*/*v*) Lutrol F-68 NF, and different concentrations of 5-ALA dissolved in citrate-phosphate buffer (pH 5.5), or 2.5% sulforhodamine B.

2.3. Optimization of coating parameters

Coating parameters, that is, (i) 5-ALA concentration in the coating solution, and (ii) the number of coating dips, were optimized so as to obtain the highest mass of 5-ALA coated on microneedle patches as well as high *in vitro* delivery efficiency in to skin. First, the concentration of 5-ALA in the coating solution was optimized by dipping each microneedle 5 times in a coating solution containing 20, 25, 30, or 50% *w*/*v* of 5-ALA, and measuring the resultant mass of 5-ALA coated on microneedles along with the delivery efficiency in skin (see 2.4). Subsequently, the number of coating dips were optimized by fixing the concentration of 5-ALA in the coating solution (obtained from the previous optimization step), and changing the number of dips at 5, 7, and 10. Again, mass of 5-ALA coated on microneedles and delivery efficiency was used for optimization.

2.4. Characterization of 5-ALA microneedles

2.4.1. Quantification of 5-ALA coated on a microneedle patch

Mass of 5-ALA on a microneedle patch was estimated using fluroscamine-based fluorescence assay. This assay involves derivatization of 5-ALA into a fluorescent compound using fluroscamine [39]. Briefly, fluroscamine reagent (0.1% *w*/*v*) was prepared by dissolving in acetonitrile. 5-ALA samples were prepared by vortexing the microneedle patches with 500 μl of citrate-phosphate buffer (pH 5.5) for 5 min. Derivatization reaction was carried out by thoroughly mixing 5-ALA samples (200 μl) with 200 μl fluroscamine reagent (0.1% *w*/*v*) and 800 μl of 0.1 M borate buffer (pH 8). The resultant solution was allowed to react at room temperature for 10 min. The fluorescent derivative of 5-ALA was analyzed using a fluorescent spectrophotometer (Cary Eclipse, Agilent Technologies, Santa Clara, CA, USA) at excitation and emission of 395 nm and 480 nm, respectively. A standard curve was obtained by derivatizing known concentrations of 5-ALA.

2.4.2. In vitro delivery efficiency in porcine skin

Full thickness porcine skin pieces from abdominal region were obtained from Innovative Research Inc. (MI, USA) and immediately stored at −80 °C. Before the experiment, skin pieces were thawed for 1 h at room temperature. Hair were carefully removed using hair clippers and the skin surface was wiped carefully with deionized water and dried completely before use. Skin pieces were placed on a fixed platform. A set of 5-ALA microneedle patches ($n = 3$) were manually inserted into the skin for 5 min and removed. Subsequently, a presoaked (by dipping in 100 μl of phosphate-citrate buffer; pH 5) cotton swab was rubbed carefully on the skin to collect 5-ALA left on the skin after insertion of patches. Immediately after, the swab was immersed into 500 μl of phosphate-citrate buffer and vortexed for 5 min. The mass of 5-ALA left on the skin surface was determined using fluorescence derivatization as discussed in the previous section. In a similar manner, the mass of 5-ALA left on microneedle patches after insertion was determined by vortexing the patches in 500 μl of phosphate-citrate buffer (pH 5), and using fluorescence derivatization to measure 5-ALA in the solutions. The delivery efficiency was calculated using the following

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