European Journal of Pharmaceutics and Biopharmaceutics 84 (2013) 297–308

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

The effect of formulation on the penetration of coated and uncoated zinc oxide nanoparticles into the viable epidermis of human skin *in vivo*

Vânia R. Leite-Silva [a,b,1], Marina Le Lamer [a,c,1], Washington Y. Sanchez [a,1], David C. Liu [d], Washington H. Sanchez [a], Isabel Morrow [e,f], Darren Martin [e], Heron D.T. Silva [b], Tarl W. Prow [d], Jeffrey E. Grice [a], Michael S. Roberts [a,g,*]

[a] *Therapeutics Research Centre, The University of Queensland, Woolloongabba, Australia*
[b] *Instituto de Ciências Ambientais Químicas e Farmacêuticas, Universidade Federal de São Paulo, Diadema SP, Brazil*
[c] *Faculty of Pharmacy, Claude Bernard University, Lyon, France*
[d] *Dermatology Research Centre, The University of Queensland, Brisbane, Australia*
[e] *ARC Centre of Excellence for Functional Nanomaterials, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia*
[f] *Australian Microscopy and Microanalysis Research Facility, The University of Queensland, Brisbane, Australia*
[g] *University of South Australia, School of Pharmacy and Medical Science, South Australia, Australia*

ARTICLE INFO

Article history:
Available online 28 February 2013

Keywords:
Zinc oxide
Nanoparticle
Skin
Multiphoton microscopy
Fluorescence lifetime

ABSTRACT

The use of nanoparticulate zinc oxide (ZnO-NP) in sunscreens and other cosmetic products has raised public health concerns. The two key issues are the extent of exposure to ZnO-NP and the likely hazard after the application of ZnO-NP in sunscreen and cosmetic products to humans *in vivo*. Our aims were to assess exposure by the extent of ZnO-NP penetration into the viable epidermis and hazard by changes in the viable epidermal redox state for a number of topical products. Of particular interest is the role of the particle coating, formulation used, and the presence of any enhancers. Multiphoton tomography with fluorescence lifetime imaging microscopy (MPT–FLIM) was used to simultaneously observe ZnO-NP penetration and potential metabolic changes within the viable epidermis of human volunteers after topical application of various ZnO-NP products. Coated and uncoated ZnO-NP remained in the superficial layers of the SC and in the skin furrows. We observed limited penetration of coated ZnO-NP dispersed in a water-in-oil emulsion formulation, which was predominantly localized adjacent to the skin furrow. However, the presence of ZnO-NP in the viable epidermis did not alter the metabolic state or morphology of the cells. In summary, our data suggest that some limited penetration of coated and uncoated ZnO-NP may occur into viable stratum granulosum epidermis adjacent to furrows, but that the extent is not sufficient to affect the redox state of those viable cells.

1. Introduction

The application of sunscreen for skin photoprotection is a key health strategy promoted by dermatologists and medical organizations to prevent ultraviolet (UV)-induced skin damage. Exposure to UV radiation is responsible for various harmful effects including sunburn, photoallergy, accelerated aging with the premature appearance of wrinkles, and an increased risk of developing a skin cancer [1,2].

Sunscreen products contain various chemical (organic) or physical (inorganic) UV filters to protect against UV radiation. Physical sunblocks include titanium dioxide (TiO_2) and zinc oxide (ZnO), as micro- and nanoparticles. Nanoparticle sunscreens offer the advantage of being transparent to light, in contrast to the opaque, white

Abbreviations: a.u., arbitrary unit; CCT, caprylic/capric triclyceride; E1, emulsion 1; E2, emulsion 2; FLIM, fluorescence lifetime imaging microscopy; fs, femtosecond; MPT, multiphoton-excited photoluminescence; MPT–FLIM, multiphoton tomography with fluorescence lifetime imaging microscopy; NA, numerical aperture; NAD(P)H, reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate; NP, nanoparticles; o/w, emulsion oil-in-water; ps, picosecond; ROS, reactive oxygen species; SC, Stratum corneum; SD, standard deviation; SG, stratum granulosum; SS, stratum spinosum; TCSPC, time correlated single-photon counting; TEWL, transepidermal water loss; UV, ultraviolet; VEC, viable epidermal cells; w/o, emulsion water-in-oil; ZnO, zinc oxide; ZnO-NP, zinc oxide nanoparticle.

* Corresponding author. The University of Queensland, Therapeutics Research Centre, School of Medicine, Princess Alexandra Hospital, Woolloongabba, Queensland, Australia. Tel.: +61 7 3176 2546.
E-mail address: m.roberts@uq.edu.au (M.S. Roberts).

[1] These authors contributed equally to this work.

0939-6411/$ - see front matter Crown Copyright © 2013 Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejpb.2013.01.020

color seen with microparticles [1]. ZnO nanoparticles (ZnO-NP) are widely used and transmit, reflect and scatter the visible part of solar radiation, while at the same time, they strongly absorb in the UV region, covering UVB (290–320 nm) as well as short (320–340 nm) and long (340–400 nm) UVA spectrum [1–3]. It has been suggested that sunscreens with larger particles of ZnO that form an opaque white film on the skin are unappealing to consumers and lead to lower and insufficient application rates [1]. Formulations with ZnO-NP are easier to spread on the skin surface and are transparent, thus providing better acceptance and compliance.

Although nanomaterials represent a major technological advance, their use raises questions from the scientific community, regulatory agencies, and the general public regarding their safety [4–6]. Concern has been raised about their increased reactivity of NPs due to their small size and their higher specific surface area [7]. Indeed, toxic effects are higher when nanoparticle surface-to-volume ratio is increased, and there is direct contact to viable cells [8,9]. However, no toxicity has been reported for topically applied nanoparticles used in sunscreens. There is also a greater propensity for smaller nanoparticles to release free radicals when exposed to light [8], but this can be reduced by coating the nanoparticle [3]. Such a coating may also improve the formulation characteristics of the nanoparticles [2].

The barrier function of the skin, generally attributed to the stratum corneum (SC), prevents the entrance of foreign molecules and pathogens from the external environment as well as the loss of endogenous substances. The skin barrier function arises from the unique lipid composition of the SC and by its organization of the orthorhombic packed intercellular lipid bilayers in multiple lamellar structures [10]. The potential of solid NP to overcome the skin barrier, to penetrate the SC, and diffuse into underlying structures lies at the center of the debate about their safe topical use in cosmetics. So far, most investigations concerning ZnO-NP have been carried out *in vitro* [9,11–14], with our group undertaking the first *in vivo* human studies [12,15,16]. Almost all reports showed that the penetration of topically applied ZnO-NP was limited to the superficial layers of the SC and that no NP was detected in the viable epidermis. However, Cross et al. [13], while showing no penetration of ZnO-NP through human skin, did show a trend to high zinc ion penetration for topically applied ZnO-NP relative to controls. A similar was reported by Gulson et al. [17,18] who detected increased levels of ^{68}Zn in blood and urine of human volunteers after topical application of sunscreens containing 19 nm ZnO particles enriched with the stable isotope ^{68}Zn under "in-use" conditions. However, it was not possible to determine whether the ^{68}Zn had penetrated the SC barrier in the form of solid particles or had previously solubilized. It is to be noted that the mean zinc recovery after ZnO-NP penetration through human skin is almost an order of magnitude less than pig skin, although the latter suggests their penetration was not significantly different to controls [14].

We previously measured the penetration of ZnO-NP into the skin by combining multiphoton tomography with spectral imaging [12]. However, the sensitivity in quantifying ZnO-NP can be increased by combining multiphoton tomography with fluorescence lifetime imaging microscopy (MPT–FLIM) [15,19]. This method uses the different "lifetimes," the average fluorescence time (photon emission) for a molecule arising from it returning from an excitation light induced excited state to its ground state. Fluorescent lifetime imaging (FLIM) is the resolution of lifetimes for different molecules in space (x,y,z) and time [19]. FLIM can also be combined with spectral imaging, a process that has been called Multispectral FLIM (SLIM) detection [20]. Thus, FLIM and SLIM can be used to produce a spatial image showing the differences in the fluorescence lifetime between several fluorophores and can therefore distinguish compounds with a similar emission spectrum [21]. MPT differs from traditional confocal fluorescence microscopy by using two or more photons of low energy, rather than one high-energy photon, to excite fluorophores within the sample. In this case, the excited state of the molecule is reached when it absorbs two photons simultaneously. Since the energy of the photons is inversely proportional to the wavelength, longer excitation wavelengths (typically infrared) can be used compared to single-photon microscopy. As these causes less photodamage than short wavelength lasers, MPT is well-suited for imaging live cells [22]. At the same time, deeper tissues can be imaged, due to the greater penetration of infrared radiation [23,24].

Reduced nicotinamide adenine dinucleotide (NADH), reduced NADH phosphate (NADPH), and flavine adenine dinucleotide (FAD) are the major autofluorescent metabolic species in skin. As NADH and NADPH share overlapping absorption, fluorescence, and lifetime properties, both molecules are collectively referred to as NAD(P)H. However, most of the NAD(P)H fluorescence (up to tenfold) is believed to arise from NADH [25]. Changes in NAD(P)H and FAD intensities and fluorescence lifetimes are linked to alterations in the cellular metabolic state [25]. NADH is a central coenzyme found in all living cells and is involved in several energy metabolic pathways, such as anaerobic glycolysis, the electron transport chain, and the citric acid cycle. FAD is a redox cofactor used for electron transport and has a key role in oxidative phosphorylation [25]. NADH and FAD^+ provide different measures of the oxidation state of a cell in that NADH and NADPH increase in intensity on the depletion of oxygen, whereas the intensity of FAD^+ is increased by oxygen [26]. Their conjugate species, NAD^+, $NADP^+$, and FADH, are non-fluorescent [25].

The aim of our study was to evaluate the influence of coating and formulation on ZnO-NP penetration into the different skin layers of human volunteers using the noninvasive MPT–FLIM technique. Three different types of formulations were examined: a gel, an oil-in-water emulsion (o/w; E1), and a water-in-oil emulsion (w/o; E2). Concordantly, NAD(P)H and FAD lifetimes were analyzed to monitor potential changes in the metabolic activity of the cells treated with ZnO-NP formulations.

2. Materials and methods

2.1. ZnO nanoparticles

In this study, two different kinds of ZnO-NP used in commercial cosmetic products were compared: Z-COTE® and Z-COTE® HP1 (BASF, Ludwigshafen, Germany). Both are inorganic micronized pigments in the form of a dry white powder. Z-COTE® are uncoated nanoparticles which have an amphiphilic nature but are preferentially incorporated into the water phase of a formulation. Z-COTE® HP1 consists of approximately 98% of ZnO and 2% of triethoxycaprylylsilane, which is a silicone derivative, used as a hydrophobic coating material. This coated ZnO-NP is therefore hydrophobic and can easily be dispersed into the oil phase of a formulation. According to the manufacturer's specifications, both coated and uncoated nanoparticles are less than 200 nm in size.

2.2. Formulations

Three vehicles were prepared: a gel, an o/w emulsion (E1), and a w/o emulsion (E2). The composition of each formulation is listed in Table 1. In each case, ingredients are defined according to the INCI (International Nomenclature of Cosmetic Ingredients) convention, quantities are listed as percentages, and triethanolamine was used to adjust the formulation pH to be between 5.5 and 6. The E1 and E2 formulations both contained the hydrating agents: glycerin (which is also present in the gel formulation), lanolin and