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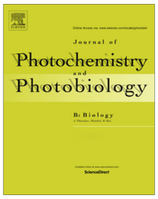

Detecting free radicals in sunscreens exposed to UVA radiation using chemiluminescence

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ABSTRACT

One of the current concerns with the application of nanoparticles in sunscreens, and in particular nano-TiO_2 and ZnO, is their potential to photogenerate free radicals and reactive oxygen species (ROS) when they absorb ultraviolet wavelengths from sunlight. Free radicals and ROS are known to be associated with UV-induced skin damage and oxidative stress, from which sunscreens are expected to offer significant protection. Here we describe a simple method, based on chemiluminescence emission, for detecting free radicals generated in commercial sunscreens alone, and when applied to various substrates, following exposure to UVA (320–400 nm) radiation. This photo-induced chemiluminescence (PICL) technique could be used to optimise sunscreen formulations so as to minimise free radical photogeneration during exposure to sunlight.

1. Introduction

One mechanism of ultraviolet (UV) radiation-mediated skin damage involves the formation of free radicals and reactive oxygen species (ROS) which rapidly deplete the natural antioxidant capacity of the skin, causing oxidative stress [1,2]. ROS include singlet oxygen, superoxides, peroxides, hydroperoxides and hydroxyl radicals. The broad spectrum UV filters present in modern sunscreens are designed to absorb UV radiation across the entire solar UV range. Two types of UV filter are used in sunscreens, chemical and physical. Chemical filters are solvated discrete molecules or molecular agglomerates that absorb UV wavelengths, whereas physical filters are dispersed inorganic particles, most commonly titanium dioxide (TiO_2) and zinc oxide (ZnO), which can effectively reflect and absorb UV radiation. Nanoparticles described as 'ultrafine' or 'micronized' (where at least one dimension is less than 100 nm) are more effective at scattering UV radiation than larger particles, are transparent on the skin, and are therefore preferred for modern sunscreen formulations [3].

Measurements of the absorption spectra of thin films of sunscreen show that both types of filter attenuate UV wavelengths, reducing the flux of UV photons reaching the surface of the skin. However recent studies have raised concerns about the effects on the skin of free radicals and reactive oxygen species (ROS) that may be photogenerated by the sunscreens themselves. Studies on some organic chemical filters applied to *ex vivo* skin have shown that higher levels of free radicals are produced than on untreated controls after irradiation for periods of one hour or more [4]. Commercial sunscreens containing TiO_2 have been shown to produce ROS, with TiO_2 causing damage to DNA in cells *in vitro*, as a result of photocatalysis [5,6]. An *in vivo* study has shown a large reduction in the collagen content of mouse skin exposed to topically-applied nano-TiO_2 over a prolonged period [7]. Remarkably, certain nanoparticle-containing sunscreens are also responsible for free-radical photocatalytic degradation of surface coatings on steel roofing materials [8]. Residues of sunscreen worn by workers who carried out the installation were left on the surface of roofing panels near the panel edges. Affected areas of the coating underwent weathering by sunlight accelerated by a factor of ~100, revealing a series of fingerprints etched into the coating [8].

It is well known that both TiO_2 (in particular the anatase form) and ZnO are semiconductors and act as photocatalysts that can be used for oxidising organic materials [9]. The mechanism involves absorption of UVA radiation at the particle surface and promotion of an electron across the band gap into the conduction band of the semiconductor, leaving a positively charged hole in the lattice [9]. In most cases the hole and electron rapidly recombine, but the

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probability of charge separation increases as particle size decreases, and is favoured by certain lattice structures. Under aqueous conditions, the electron can react with oxygen to form a superoxide radical anion, and the hole with water to form a hydroxyl radical and a proton [9]. Hydrogen peroxide can be formed from both radical products, and photogeneration of singlet oxygen has also been reported. Excess ROS are well known to have cytotoxic and genotoxic properties [10]. The rate of photocatalysis increases with decreasing particle size from the micrometre to the nanometre scale, due to the larger surface area to mass ratio for nanoparticles [11]. Similarly, the clustering of metal oxide nanoparticles into agglomerates and/or aggregates in liquid dispersions reduces their photocatalytic activity [12].

It is possible to reduce the photocatalytic activity of TiO_2 and ZnO nanoparticles by applying a surface coating or by doping their internal structures with small amounts of other materials. Typical coatings used that do not affect the UV absorption properties of the nanoparticles are inorganic oxides, such as silica and alumina, or polymer coatings, such as polymethyl methacrylate (PMMA) [13,14]. Examples of dopants that reduce photocatalytic activity include Mn, Co and Ni for ZnO [15] and Mn and carbonaceous residues for TiO_2 [16,17]. However a recent study [18] showed that despite coating or doping, TiO_2 nanoparticles retained a significant level of photocatalytic activity, particularly those containing the anatase form.

Whilst there are a number of test methods for assessing the photocatalytic activity of nanoparticles, most of which measure the rate of bleaching of coloured dyes [19,20], direct detection of free radicals formed in skin exposed to sunlight is far more difficult. The only direct method for studying free radicals is electron spin resonance (ESR) spectroscopy, which requires access to a specialised and expensive instrument. Most ESR instruments operate at 8–10 GHz (X-band) and have moderate to low sensitivity. The free radicals formed in irradiated skin have a short lifetime, and therefore it is necessary to use spin trapping agents to form more stable radicals to study [21]. An ESR spin-trapping method has been described to determine the presence of free radicals in irradiated sunscreens and this has recently been applied to establish the beneficial effect of antioxidants in the formulation [22,23].

A convenient alternative to ESR is to measure the chemiluminescence emission which is observed when free radicals react with atmospheric oxygen. Despite the very low quantum yield of chemiluminescence emission resulting from the reaction of carbon-centred free radicals with oxygen (typically 10^{-5}–10^{-13}), use of photon counting detection makes the technique extremely sensitive.

A number of groups have reported luminescence emission from skin exposed to UV radiation in air both *in vivo* and *in vitro* [24–28]. However in these reports the chemiluminescence produced by free radical reactions is not separated from photophysical light emission due to the processes of fluorescence, phosphorescence and charge recombination luminescence. We have recently published the results of a photo-induced chemiluminescence (PICL) study on bovine *stratum corneum* exposed to UVA [29]. To separate the chemiluminescence produced via the reaction of free radicals in the skin with oxygen from photophysical light emission processes, a protocol was used where the skin was irradiated with UVA in a stream of nitrogen. Photophysical luminescence was allowed to decay before switching the gas from nitrogen to oxygen, allowing free radicals formed in the irradiated skin to react with oxygen, resulting in a burst of weak PICL.

The mechanism for free radical oxidation of organic materials is shown in Scheme 1. It involves the formation of a population of carbon free radicals $P^{\bullet}$ (or radical ions) when the substrate is irradiated in an inert gas, such as N_2. These radicals remain stable in the solid material until O_2 is admitted. Oxygen is a bi-radical and reacts rapidly with the carbon free radicals to form peroxy radicals and hydroperoxides, which are known to be the precursors for chemiluminescence emission in polymers [31]. PICL emission from organic materials, first proposed by Russell [30], occurs via a dimer formed by combination of two peroxy radicals, POOOOP, as shown in Eqs. (6)–(8) in Scheme 1. The mechanism to produce an excited carbonyl (Eq. (7)) requires one of the carbon free radicals to be either primary or secondary [30].

PICL has been observed from organic polymer films and fibres [32], including fibrous proteins [33,34]. In this paper we report PICL emission from *ex vivo* UVA-exposed mouse skin, and skin treated with eight proprietary sunscreens. Nanoparticulates were extracted from seven of these sunscreens and characterised for their morphologies and sizes using transmission electron microscopy (TEM). The photocatalytic activity of the extracted particles was assessed using aqueous suspensions containing Rhodamine B exposed to UVA radiation. We also report PICL emission from the sunscreens applied to wool keratin. Keratin is the primary component of the cornified outer layer of the *stratum corneum*, and wool keratin is a convenient material for PICL studies on sunscreens due to its higher intensity PICL emission compared with skin. PICL emission was also measured from the sunscreens themselves in the absence of any substrate. Finally, some laboratory formulated sunscreens containing a variety of different TiO_2 and ZnO nanoparticles were prepared and examined for PICL using Whatman 54 filter paper as a convenient carrier substrate.

2. Materials and methods

2.1. Mouse skin samples

Female, immune-competent, hairless SKH:QS mice (6 months old) were supplied in-house by the CSIRO Animal House from an in bred colony. Animal experiments were conducted following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004), and were approved by the CSIRO Animal Ethics Committee (ACEC 08-17). Prior to use, mice were housed in groups of ten in open-topped polycarbonate cages in an isolated temperature (∼21 °C) and moisture (55–65% relative humidity) controlled room with a 14 h light/10 h dark cycle. Mice had *ad libitum* access to water and Gordon's rat and mouse pellets (Gordon's Speciality Stock Feeds, Australia).

Mice were anaesthetised (intraperitoneal injection of Xylase ($50\ mg\ kg^{-1}$)/ketamine ($50\ mg\ kg^{-1}$)) and then killed by cervical dislocation. Mouse skins were carefully excised, placed dermis-side down onto backing card, and stored at −80 °C until use.

2.2. Sunscreens

Samples of eight proprietary sunscreens, including sunscreens containing ZnO and TiO_2, were sourced from local retail outlets in Australia. In addition, some laboratory sunscreens containing inorganic UV absorbers were prepared using a basic formulation, as previously described [35]. Briefly, dispersions (100 g) were prepared using castor oil (85 g, Sigma Aldrich), isopropyl myristate (12 g, Sigma Aldrich) and nanoparticulate metal oxide (3 g), which were mixed for 5 min in a narrow vessel using a laboratory homogenizer (Model SEV, Kinematica AG, Lucerne, Switzerland) on speed setting 4. These dispersions were applied to pre-weighed discs of Whatman 54 filter paper for PICL studies immediately after homogenization.

Nanomaterials used to prepare laboratory sunscreen formulations were P25 TiO_2 (Degussa) which consists of a mixture of anatase and rutile phases in the ratio 3:1 [36], nano-anatase (Sigma Aldrich, cat. no. 637254), nano-rutile (Sigma Aldrich, cat. no. 637262),