

## Note

## Prevention of UV-Induced Melanin Production by Accumulation of Redox Nanoparticles in the Epidermal Layer *via* Iontophoresis

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UV rays induce melanin production in the skin, which, from a cosmetic point of view, is problematic. Reactive oxygen species (ROS) generated in the skin upon UV irradiation are thought to be responsible for melanin production. Thus, effective antioxidants are recognized as useful tools for prevention of UV-induced melanin production. Redox nanoparticles (RNPs) containing nitroxide radicals as free radical scavengers were previously developed, and shown to be effective ROS scavengers in the body. RNPs are therefore expected to be useful for effective protection against UV-induced melanin production. However, as the sizes of RNPs are typically larger than the intercellular spaces of the skin, transdermal penetration is difficult. We recently demonstrated effective transdermal delivery and accumulation of nanoparticles in the epidermal layer *via* faint electric treatment, *i.e.*, iontophoresis, suggesting that iontophoresis of RNPs may be a useful strategy for prevention of UV-induced melanin production in the skin. Herein, we performed iontophoresis of RNPs on the dorsal skin of hairless mice that produce melanin in response to light exposure. RNPs accumulated in the epidermal layer upon application of iontophoresis. Further, the combination of RNPs with iontophoresis decreased UV-induced melanin spots and melanin content in the skin. Taken together, we successfully demonstrated that iontophoresis-mediated accumulation of RNPs in the epidermis prevented melanin production.

**Key words** iontophoresis; redox nanoparticle; melanin production; reactive oxygen species; nanomedicine

Skin is exposed to UV light rays comprised of UVA (315–400 nm) and UVB (280–315 nm) rays on a daily basis. UV irradiation is known to induce various types of damage to the skin, such as DNA alterations, inflammation, and oxidative alterations of collagen and melanin production.<sup>1)</sup> Production of reactive oxygen species (ROS), such as singlet oxygen and superoxide anion, is thought to be one cause of UV-mediated skin damage.<sup>1)</sup> Thus, various antioxidants have been developed for protection of skin from ROS generated *via* UV irradiation.<sup>2)</sup> For example, carotenoid astaxanthin is known as an effective antioxidant,<sup>3–5)</sup> and we previously reported its protective effects on UV-mediated skin damage.<sup>6)</sup> However, the protective effects of such low molecular weight (LMW) antioxidants would be reduced by diffusion in the skin following topical application. Accumulation of antioxidants without diffusion in the skin would therefore be beneficial for effective protection from UV irradiation. Further, such LMW antioxidants are known to exhibit the ability to cause dysfunction of normal redox reactions (*e.g.*, the electron transport chain) in healthy cells upon internalization due to their sizes, which suppresses enough dosage to the protection of UV-mediated skin damage. We thus need to develop new antioxidants that can reduce adverse side effects to the healthy cells.

We previously reported the successful accumulation of nano-sized particles in the epidermal layer of mouse skin by iontophoresis.<sup>7)</sup> Iontophoresis is an effective technology for transdermal delivery of nanoparticles, such as liposomes, using faint electricity.<sup>8)</sup> We found that faint electric treatment opened intercellular junctions in the skin.<sup>8)</sup> Further, liposomes were shown to penetrate to deep regions of the skin through

the intercellular space by iontophoresis because of the flexible structure of the lipid vesicles.<sup>8)</sup> On the other hand, polymer-based nanoparticles accumulated in the epidermis due to their rigid structures.<sup>7)</sup> Thus, polymer-based nanoparticles that exhibit antioxidative activity may be useful for effective prevention of UV-mediated skin injury.

Recently, antioxidative polymer-based nanoparticles modified with the radical scavenger 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), referred to as redox nanoparticles (RNPs), were developed for prevention of diseases caused by oxidative stress, such as periodontal disease and colitis<sup>9,10)</sup> (Fig. 1). Due to their size of RNPs (*ca.* 30–40 nm), they hardly internalize in healthy cells to prevent unwanted adverse effects. Further, RNPs were expected to show effective prevention of UV-mediated skin damage due to their antioxidative capabilities. Since ROS are generated in the skin by UV irradiation, topical application of RNPs on skin surface would not be effective. Thus, iontophoretic delivery of RNPs is a useful strategy for scavenging ROS in the skin.

In the present study, we examined the effect of iontophoretic delivery of RNPs to the epidermal layer on UV radiation-induced melanin production, in an effort to develop an effective prevention system against UV-mediated skin damage.

### MATERIALS AND METHODS

**Animals and Materials** Male Hos:HRM2 hairless mice, aged 4–5 weeks, were purchased from Shimizu Laboratory Supplies Company, Ltd. (Kyoto, Japan). All mice were maintained and used in accordance with the animal protocol

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approved by the Institutional Animal Care and Use Committee, Kyoto Pharmaceutical University (Kyoto, Japan). The electrodes for iontophoresis (TCTTM) were manufactured by TTI Ellebeau Inc. (Tokyo, Japan). Fontana–Masson staining reagent was purchased from Muto Pure Chemical Company, Ltd. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

**Preparation of RNPs** RNPs were prepared according to a previously described method.<sup>9)</sup> Briefly, RNPs were prepared from amphiphilic block copolymer (MeO-PEG-*b*-PMNT) composed of a hydrophilic poly(ethylene glycol) (PEG) segment and a hydrophobic poly(chloromethylstyrene) (PCMS) segment, in which the chloromethyl groups were converted to nitroxide radicals, 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO), *via* the amination of MeO-PEG-*b*-PCMS block copolymer with 4-amino-TEMPO. To prepare the RNPs, MeO-PEG-*b*-PMNT was dissolved in *N,N*-dimethylformamide, and the polymer solution was transferred into a membrane tube (Spectra/Pro, molecular-weight cutoff size 3500; Spectrum, U.S.A.) and dialyzed for 24 h against water. The diameters of the resultant RNPs were measured by dy-

namic light scattering.

**Iontophoresis of RNPs** Iontophoresis of RNPs was performed as previously reported.<sup>6)</sup> Nonwoven fabric (3 cm<sup>2</sup>) containing 200  $\mu$ L of the cationic RNP suspension was placed on the dorsal skin of Hos:HRM2 hairless mice, which can easily generate melanin in response to light exposure. The nonwoven fabric containing RNPs was covered with electrode patch. The electrode patch was connected to the anode, and the saline patch to the cathode of a power supply (model-TCCR-3005; TTI Ellebeau Inc.) using Ag–AgCl electrodes. Iontophoresis was carried out at a constant current density of 0.4 mA/cm<sup>2</sup> (1.26 mA) for 45 min. This treatment was performed 3 times every 3 d during the period of UV irradiation.

**Observation of RNPs in the Skin after Iontophoresis** Following iontophoresis of RNPs labeled with the fluorescent dye rhodamine on the dorsal skin of mice, the skin was excised. Cross sections of the skin (12  $\mu$ m thickness) were prepared using a cryostat (CM1100; LEICA, Wetzlar, Germany). The skin cross sections were observed using a LSM 510 META confocal laser scanning microscope (Carl Zeiss Co., Ltd., Germany) equipped with an objective lens (EC Plan-Neofluar 10 $\times$ 0.3 M27).

**UV Treatment of Mouse Dorsal Skin** The dorsal skin of Hos:HRM2 hairless mice was irradiated with UV light (230 mJ/cm<sup>2</sup>) once a day for 5 consecutive days using a handheld UV lamp (irradiation wavelength: 302 nm, 3UV-38 3UV Lamp; UVP).<sup>6)</sup> A higher dose of UV irradiation (450 mJ/cm<sup>2</sup>) was then administered for 5 consecutive days. Iontophoresis of the RNPs was performed 3 times every 3 d before daily UV irradiation (Fig. 2).

**Evaluation of Melanin Production in the Skin after UV Irradiation** For detection of melanin in the skin after UV irradiation for 10 d, each specimen soaked in formaldehyde was stained by the Fontana–Masson staining method.<sup>6)</sup> Skin cross sections after Fontana–Masson staining were observed with light microscopy to detect melanin spots. To quantify melanin content in the skin, a piece of the skin was washed 3 times with phosphate buffered saline and then additionally washed twice with ethanol–ether (3:1) and once with ether. The piece of skin was then added to 1 mL of a 2 M NaOH solution, and the mixture was heated at 80°C for solubilization. After cooling, absorbance of the solubilized sample was measured at 475 nm to evaluate melanin content.<sup>6)</sup>

**Statistical Analysis** Statistical significance was determined using the Student's *t*-test. A *p* value <0.05 was considered to be significant.

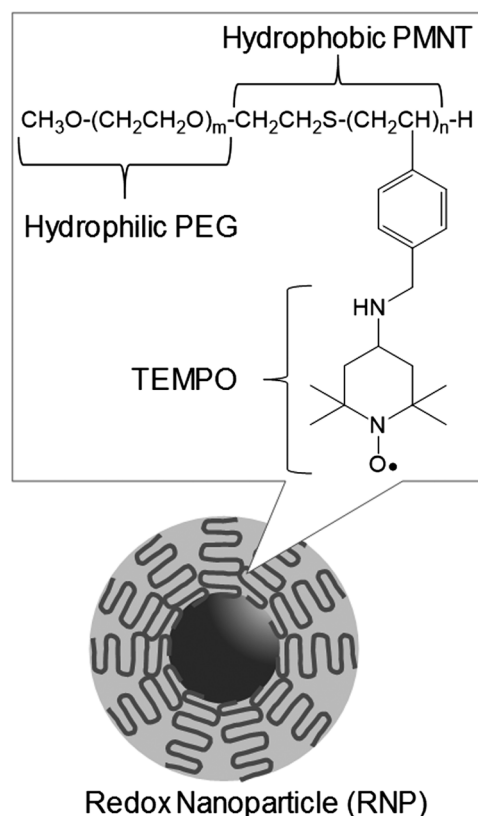


Fig. 1. Structure of MeO-PEG-*b*-PMNT and Schematic of Redox Nanoparticles (RNPs) Comprised of MeO-PEG-*b*-PMNT

## RESULTS AND DISCUSSION

### Accumulation of RNPs in the Epidermis by Iontophoretic Delivery

The diameter and zeta-potential of the RNPs

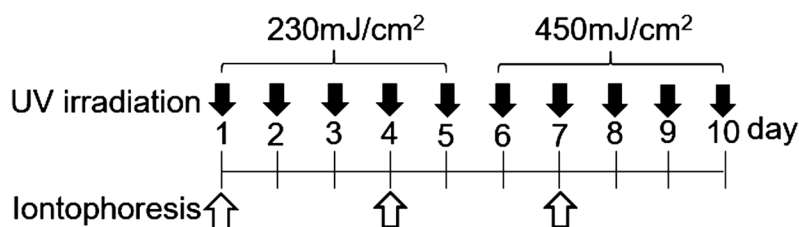


Fig. 2. Treatment Schedule of UV Irradiation and Iontophoresis



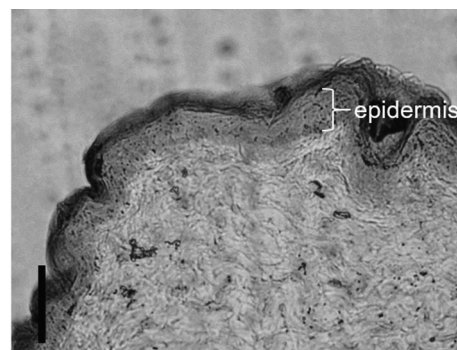
Fig. 3. Accumulation of RNPs in the Epidermis after Iontophoresis

After iontophoresis of RNPs labeled with rhodamine on the dorsal skin of hairless mice, skin cross-sections were prepared and observed by confocal laser scanning microscopy. The upper panel shows the transmitted light image, while the lower panel shows the fluorescence image. The scale bar indicates 50  $\mu\text{m}$ .

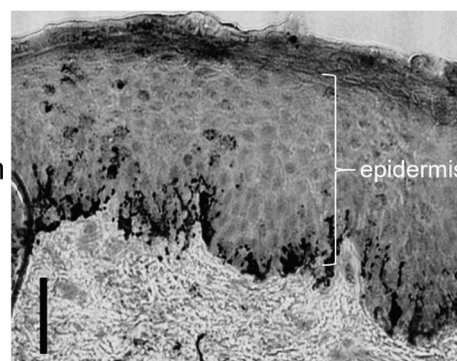
were *ca.* 40 nm and 7.4 mV, respectively. As the surface charge of the RNPs was positive, the anodal iontophoresis was performed on the dorsal skin of hairless mice. Cross sections of skin following iontophoresis of fluorescently-labeled RNPs were observed by confocal laser scanning microscopy. As expected, the fluorescence signals were clearly observed in the epidermis (Fig. 3). It should be noted that no fluorescence was observed in the dermis, which is an ideal characteristic of cosmetics. Accumulation of RNPs in epidermis is probably due to their rigid structures, although liposomes having flexible property penetrated to deep region of the skin through narrow intercellular space.<sup>8)</sup> Following confirmation of the accumulation of RNPs in the epidermis upon application of the iontophoresis technique, we subsequently evaluated the protective effect of RNPs on UV-induced melanin production.

**Effect of RNP Iontophoresis on UV-Induced Melanin Production** Iontophoresis of RNPs was performed 3 times every 3 d before daily UV irradiation to promote accumulation of RNPs in the epidermis (Fig. 2). Hypertrophy of the epidermis was observed and the number of black spots, which denote melanin pigment lumps, increased in the UV-irradiated skin, while the epidermis of untreated skin was thin and contained few black spots (Fig. 4). Levels of melanin increased by a factor of 1.3 following treatment with UV irradiation (Fig. 5). However, the number of black spots decreased in skin treated with RNP iontophoresis during UV irradiation (Fig. 4), and levels of melanin decreased upon RNP iontophoresis. The suppression efficiency of UV-mediated melanin production was about 50% (Fig. 5). Level of melanin in skin treated by RNP iontophoresis was about 60% of control skin, although

Untreated



UV irradiation



UV irradiation  
+ RNP/IP

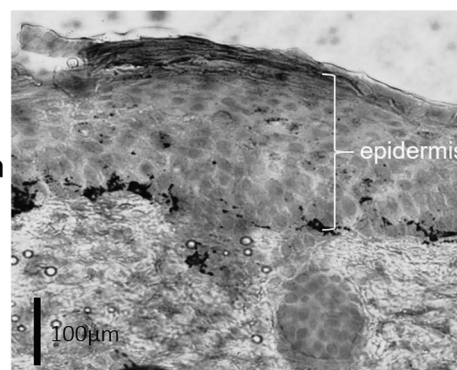


Fig. 4. Effect of RNP Iontophoresis on UV-Induced Skin Pigmentation

The light microscopic images are cross sections of control skin (Untreated), UV-irradiated skin (UV irradiation), and UV-irradiated skin treated with RNP iontophoresis (UV irradiation+RNP/IP). Scale bar indicates 100  $\mu\text{m}$ .

the difference was not statistically significant ( $p=0.057$ ). The lower amount of melanin in RNP-treated skin relative to control skin is likely due to prevention of constant melanin production by daily light exposure.

Iontophoretic transdermal delivery of astaxanthin liposomes was previously shown to effectively prevent melanin production.<sup>6)</sup> The protective effect of astaxanthin liposomes on UV-induced melanin production was about 40% (unpublished data). As stated above, RNPs selectively accumulate in the epidermis, unlike liposomes, which tend to spread to the dermis.<sup>8)</sup> Moreover, it has been reported that TEMPO scavenges ROS catalytically.<sup>9,10)</sup> Thus, high accumulation of RNPs in the epidermis, coupled with the ability to catalytically eliminate ROS, may improve the effects on UV-induced melanin production. As the epidermal layer is gradually replaced and undergoes turnover in 4–6 weeks,<sup>11)</sup> RNP accumulation in the epidermis is not expected to cause any long-term toxic effects. Taken together, iontophoresis-induced antioxidative nanothera-



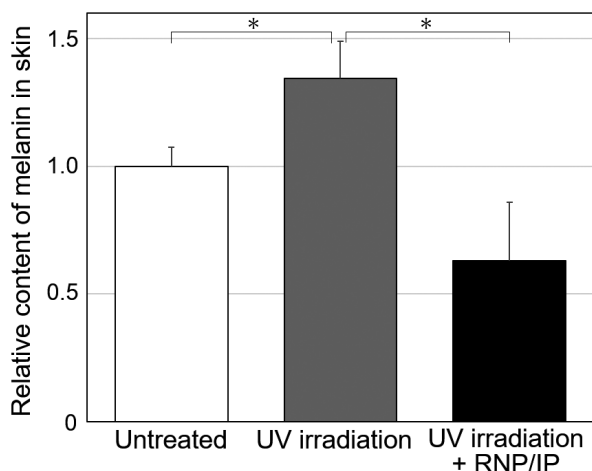


Fig. 5. Effect of RNP Iontophoresis on Melanin Content in UV-Irradiated Skin

Relative amount of melanin in the skin of control mice (Untreated), UV-irradiated mice (UV irradiation), and UV-irradiated mice pretreated with RNP iontophoresis (UV irradiation+RNP/IP) were measured as described in Materials and Methods. Data are average  $\pm$  standard deviation of the relative amount of melanin extracted from the skin of at least 3 mice in each group. \* $p < 0.05$ .

py using RNPs should serve as an effective protection system against UV-induced skin damage.

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**Conflict of Interest** The authors declare no conflict of interest.

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