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Photocytotoxicity in human dermal fibroblasts elicited by permanent makeup inks containing titanium dioxide

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Synopsis

Titanium dioxide (TiO_2) is a pigment widely used in decorative tattoo and permanent makeup inks. However, little is known about the risks associated with its presence in these products. We have developed an *in vitro* assay to identify inks containing TiO_2 that are cytotoxic and/or photocytotoxic. The presence of TiO_2 in ten permanent makeup inks was established by X-ray fluorescence. Using X-ray diffraction, we found that seven inks contained predominately TiO_2 (anatase), the more photocatalytically active crystalline form of TiO_2. The remaining inks contained predominately TiO_2 (rutile). To identify cytotoxic and/or photocytotoxic inks, human dermal fibroblasts were incubated for 18 h in media containing inks or pigments isolated from inks. Fibroblasts were then irradiated with 10 J/cm^2 UVA radiation combined with 45 J/cm^2 visible light for determining photocytotoxicity, or kept in the dark for determining cytotoxicity. Toxicity was assessed as inhibition of colony formation. No inks were cytotoxic. However eight inks, and the pigments isolated from these inks, were photocytotoxic. Using ESR, we found that most pigments from photocytotoxic inks generated hydroxyl radicals when photoexcited with UV radiation. Therefore, the possibility of photocytotoxicity should be considered when evaluating the safety of permanent makeup inks containing TiO_2.

INTRODUCTION

Tattoos have been used as a means for expressing cultural and personal identity for millennia. A more recent development is intradermal implantation of pigments as an alternative to facial makeup. The popularity of cosmetic tattooing or permanent makeup has steadily increased since its widespread introduction in the mid 1980s (1). The U.S. Food and Drug Administration (FDA) considers inks used for tattoos and permanent makeup to be cosmetics (2). The pigments used in these inks are considered to be color additives, and, therefore, are subject to premarket approval for this specific use (3). To date, no pigments have been approved by the Food and Drug Administration for use in tattoos or permanent makeup. Until recently, there have been limited reports of adverse reactions to inks. The European Commission has recognized the need to address potential risks associated with inks used for decorative tattoos and permanent makeup (4,5). In Europe, these products are not considered cosmetics but are regulated as consumer products under the restrictions of the General Product Safety Directive (5).

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The patterns of use for these products are dramatically changing. One notable change is the popularity of tattoos and permanent makeup. It has been reported that 24% of the population in the contiguous United States have at least one tattoo (6). The highest incidence of tattoos (36%) occurred for individuals aged 18 to 29. Another change in the pattern of use is the formulation of inks with novel pigments, which allows a wide range of colors for tattoos and permanent makeup. Increasingly, inks are being formulated with organic pigments that have a limited history of safe use (7–9). The high prevalence of tattoos and permanent makeup combined with the use of novel pigments increases the risk for adverse reactions to these products. As an example of this increased risk, the FDA had received only five reports of adverse reactions after permanent makeup procedures between 1988 and 2003 (10). In contrast, between 2003 and 2004, the FDA received 150 reports of adverse reactions attributable to use of inks traceable to one manufacturer of permanent makeup inks. The FDA warned consumers about the adverse reaction reports, and the inks were subsequently voluntarily withdrawn from the market (10,11).

Reliable toxicological testing methods are needed to assure the safety of tattoo and permanent makeup inks. However, there have been very few attempts to develop *in vitro* methods for identifying hazardous inks. Falconi *et al*., (12) have used human fibroblasts derived from gingival tissue to investigate the cytotoxicity of a permanent makeup ink. The cytotoxicity, resulting from exposures to the ink for up to two weeks, was assessed by measuring inhibition of the fibroblasts' capacity to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). Dose-dependent cytotoxicity was observed for the selected ink. We have developed an *in vitro* assay that allows evaluation of both the cytotoxicity and photocytotoxity of inks. Because inks used for decorative tattoos and permanent makeup contain pigments that are efficient chromophores and these inks are applied to sun-exposed skin, phototoxicity is a concern. There have been reports of phototoxic reactions resulting from decorative tattoos and permanent makeup (13–20). Although the *in vitro* assay described here was developed to assess the cytotoxicity and photocytotoxicity of permanent makeup inks, the method is generally more applicable for testing inks used for either decorative tattoos or permanent makeup.

In our initial studies, we examined the cytotoxicity and photocytotoxicity of permanent makeup inks containing TiO_2. These inks were selected for several reasons. First, TiO_2 is one of the most widely used pigments in tattoo and permanent makeup inks. Due to its high refractive index, TiO_2 frequently functions as an opacifier in inks (1). Titanium dioxide is also widely used in pigment mixtures to formulate light shades of inks. In addition, the photocatalytic activity of TiO_2 has been widely studied. Therefore, TiO_2 is an ideal pigment to investigate in the development of an *in vitro* assay for photocytotoxicity. Finally, there have been reports associating the presence of TiO_2 in inks with adverse reactions (21,22). Therefore, toxicological data for inks containing TiO_2 are needed.

EXPERIMENTAL

MATERIALS

Titanium dioxide (anatase) was purchased from Sigma-Aldrich (Milwaukee, WI). Samples of TiO_2 (rutile) were obtained from Sigma-Aldrich and Tronox (Oklahoma City, OK). Permanent makeup inks were purchased through the Internet from ten vendors.

White or lightly colored shades of inks were selected. The purchases were made between January 2006 and May 2010. All inks were explicitly labeled by the vendors for use as permanent makeup.

GRAVIMETRIC DETERMINATION OF PERMANENT MAKEUP INKS' PIGMENT CONTENT

A 50-μl aliquot of permanent makeup ink was deposited onto a preweighed inorganic membrane filter (0.02-μm pore size, 10-mm diameter; Whatman Inc., Clifton, NJ). The filter with deposited ink was weighed, and subsequently washed five times under gentle vacuum using 50 μl of distilled water. The filter was then dried for three days in a ventilated oven operating at 35° ± 2°C. The filter was then weighed to obtain the weight of the dried pigment. The weight of the dried pigment and the initial weight of the permanent makeup ink were used to calculate the percentage of pigment in the permanent makeup ink.

ISOLATION OF PIGMENTS FROM PERMANENT MAKEUP INKS

One ml of permanent makeup ink was diluted with 3 ml of deionized water and centrifuged at 85,000g (15°C) for one hour. (Optima L-90K ultracentrifuge, Beckman Coulter, Inc., Brea, CA). Sedimented pigments were washed twice by resuspension in 4 ml of deionized water and centrifugation as above. Pigments were then dried overnight under vacuum at 30°C.

ELEMENTAL ANALYSIS OF PIGMENTS FROM PERMANENT MAKEUP INKS BY X-RAY FLUORESCENCE

Each sample of pigment (approximately 200 mg) was mixed with paraffin wax and pressed in a pellet die at 30 tons for five minutes to form a standard pellet. X-ray fluorescence measurements were made using a Bruker S4 wavelength dispersive X-ray fluorescence spectrometer (Bruker AXS Inc., Madison, WI). The spectrometer sequentially searches for elements with atomic numbers from Na to U and adjusts the test conditions for each element to optimize the detection sensitivity. A semiquantitative analysis was performed using the fluorescence yield for each element and accounting for enhancements attributed to secondary excitation and absorption due to heavy elements. The semiquantitative analysis has a typical accuracy of 5%. The elements Al, Si, and Ti are reported as their most common oxides.

DETERMINATION OF THE CRYSTALLINE PHASE OF TiO_2 BY X-RAY DIFFRACTION

Pigments isolated from permanent makeup inks were loaded onto a zero background holder and placed into a Phillips PW3020 diffractometer (Phillips Electronic Instruments, Inc., Mahwah, NJ) using Cu-Kα radiation at 40 KV and 30 mA. Scans were run over the range of 20° to 80° with a step size of 0.02° and a counting time of four hours. The crystalline phase of TiO_2 was identified using the powder diffraction file published by the International Centre for Diffraction Data (Newtown Square, PA). Once all phases were identified, the amount of TiO_2 in each was quantified using a Rietveld refinement for comparing the computed diffraction pattern with the observed diffraction pattern.

CELL CULTURE

Human skin fibroblasts (ATCC CRL-1634) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's

medium, without phenol red, containing 10% fetal bovine serum, 50 μg/ml gentamicin, 4.5 mg/ml glucose, and 4 mM L-glutamine. All reagents used for cell culture were obtained from Invitrogen Corp., Carlsbad, CA. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

IN VITRO ASSAY FOR CYTOTOXICITY AND PHOTOCYTOTOXICITY

Fibroblasts were incubated for 18 hours with media containing either a permanent makeup ink or the pigment isolated from an ink. For treatment with a permanent makeup ink, a stock solution of the ink was prepared by dispersing the ink in deionized water using a ten-second ultrasonic burst (Vibra-Cell VC250B sonicator, Sonics & Materials Inc., Danbury. CT). The stock solution was then heated for ten minutes at 100°C to minimize microbial contamination. Working solutions for treating fibroblasts were prepared by appropriately diluting stock solutions with media. Using the pigment content of each ink determined by gravimetric analysis, the level of treatment was expressed as the amount of pigment contained in the ink for each treatment and is given as μg of pigment per surface area of the fibroblast monolayer. For treatment with pigments, the pigments were dispersed in deionized water as described above and diluted in media to obtain working solutions used to treat fibroblasts.

Following the 18-hour incubation in media containing a permanent makeup ink or pigment, fibroblasts were washed once with phosphate-buffered saline (PBS). Fibroblasts were then irradiated through freshly added PBS with 10 J/cm^2 UVA radiation (320 nm–400nm) combined with 45 J/cm^2 visible light (400 nm–800 nm). Similar levels of UVA radiation and visible light would be received after exposure to the summer sun for 30 minutes (23). The source of UVA radiation and visible light was a 250-watt HITLite metal halide bulb (BLV Licht-und Vakuumteenik GmbH, Steinhöring, Germany) filtered through glass. The emission spectrum of the light source was measured using an OL 754 UV-visible spectroradiometer (Optronic Laboratories Inc., Orlando, FL) and is shown in Figure 1. The spectral irradiance of the light source was found to be typically 6.3×10^{-3} W/cm^2 UVA radiation and 2.8×10^{-2} W/cm^2 visible light. The emission of UVB radiation (280 nm–320 nm) from the light source was negligible (i.e., 1.1×10^{-7} W/cm^2). All irradiations were performed at 25° ± 3°C, and lasted approximately 25 minutes for simultaneous delivery of 10 J/cm^2 UVA radiation and 45 J/cm^2 visible light. To compensate for any inhomogeneity in the field of illumination, uncovered samples were placed on a platform that rotated at 0.5 revolutions/min during irradiation. Sham-irradiated (i.e., dark control) samples were maintained at 25° ± 3°C in the dark. After irradiation, cells were removed from the dishes by trypsinization and plated into 60-mm Petri dishes (~800 cells/dish). Four replicate dishes were plated for each treatment. The dishes were then incubated for 10–14 days to allow formation of cell colonies. Colonies were fixed with methanol, stained with Giemsa stain, and counted. The average number of colonies observed after a treatment and the average number of colonies observed for cells receiving no treatment were used to calculate the percentage of cells surviving a treatment. Cytotoxicity was assessed using survival data for fibroblasts receiving treatment with an ink or a pigment alone (i.e., sham-irradiated samples), while photocytotoxicity was assessed from survival data for cells additionally exposed to light. A four-parameter logistic function (SigmaPlot 8, SPSS Inc., Chicago, IL) was used to fit the data (i.e., % survival *versus* μg/cm^2) and to determine the PD_{50} (dose of pigment which, in the presence of light, reduced survival by 50%).

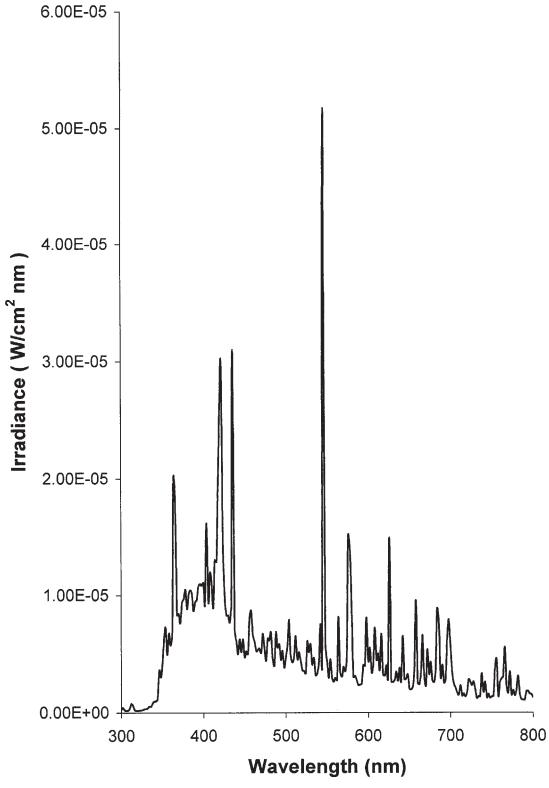

Figure 1. Emission spectrum of the light source used for assessing photocytotoxicity.

DETECTION OF FREE RADICALS FORMED FOLLOWING PHOTOEXCITATION OF PIGMENTS ISOLATED FROM PERMANENT MAKEUP INKS

Electron spin resonance spectroscopy (ESR) with spin trapping was used to detect hydroxyl radicals ($HO^{\bullet}$) formed during photoexcitation of pigments isolated from permanent makeup inks. A sample, containing 1 to 100 μg/ml of pigment suspended in water with the spin trap, 5,5-dimethyl *N*-oxide pyrroline (DMPO, 50 mM), was transferred to a quartz capillary tube. The capillary tube was placed into the microwave cavity of a Bruker EMX ESR spectrometer (Billerica, MA). Samples were irradiated with UV radiation (320 nm) in the microwave cavity, using a 500-watt Xe arc lamp directed through a McPherson monochromator, model DM200 (Chelmsford, MA). ESR spectra were collected during irradiation times from 1 to 25 minutes All ESR measurements were carried out at ambient temperature (27°C), using the following settings for detection of the spin adduct between DMPO and $HO^{\bullet}$ (DMPO-OH): 20 mW microwave power, 100 G scan range, and 1 G field modulation. An ESR spectral profile characteristic for the DMPO-OH adduct was observed and contained four lines (relative intensities of 1:2:2:1) with hyperfine splitting parameters $a^N = a^H = 14.9$ G (Figure 4).

STATISTICAL ANALYSIS

Student's *t*-test was used to determine the statistical significance of differences between the PD_{50} determined for an ink and the PD_{50} determined for the pigment isolated from the ink. *P*-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

CHARACTERISTICS OF THE PIGMENTS IN PERMANENT MAKEUP INKS

The permanent makeup inks studied were white, except for inks 4 (flesh-colored), 5 (light beige), and 9 (flesh-colored). A partial characterization of pigments occurring in the permanent makeup inks is given in Table I. The pigment content of the inks varied between 32.8 and 67.8 w/w%. Analysis by X-ray fluorescence indicated that the principal component of the pigments in all ten inks was TiO_2 (85.9 to 99.8 w/w%). In addition, several inks contained Al and Si, which are reported in Table I as their most commonly occurring oxides, alumina and silica, respectively. These inorganic oxides are frequently used as surface coatings, applied both to reduce the photocatalytic activity of TiO_2 and to facilitate incorporation of TiO_2 into formulations by reducing particle aggregation (24). Alumina may also be added to promote the formation of TiO_2 (rutile) during the production of TiO_2 (24). Two of the colored inks, inks 5 (light beige) and 9 (flesh-colored), contained iron oxide. Iron oxide is frequently used in mixtures of pigments to provide shades of brown (25,26). Ink 4 contained 0.3 % chlorine. Two elements, niobium (Nb) and P, were present in trace amounts (<0.1%). These elements occur in ore used for production of TiO_2, and therefore low levels of these elements are frequently found in commercially available TiO_2 (24).

Numerous studies have shown that the photocytotoxicity of TiO_2 results from its ability to photocatalyze the generation of reactive oxygen species (ROS). Titanium dioxide strongly absorbs radiation in the ultraviolet A spectral region (320–400 nm) (27). Due to its semiconductor properties, photoexcitation of TiO_2 with UVA radiation results in charge separation, i.e., creation of electron-hole pairs, within particles of TiO_2 (28,29). These paired charges can either recombine or ultimately react with water or oxygen near the particle's surface to form ROS. The ROS formed include hydroxyl radical, superoxide radical anion, hydrogen peroxide, and singlet oxygen (30–32). The ROS generated in this photocatalytic cycle can then damage cellular components, including membranes and

Table I
Pigment Content and Elemental Composition of Inks

Sample	Pigment content of ink* (w/w %)	Composition of pigment[a] (w/w %)
Ink 1	33.0 ± 0.8	TiO_2 (99.7), P (tr[b])
Ink 2	44.9 ± 1.8	TiO_2 (99.2), Al_2O_3 (0.2), SiO_2 (0.2), P (tr)
Ink 3	38.1 ± 1.4	TiO_2 (99.6), P (tr)
Ink 4	52.5 ± 1.2	TiO_2 (95.8), Al_2O_3 (2.6), SiO_2 (0.8), CI (0.3), P (tr)
Ink 5	57.5 ± 2.9	TiO_2 (98.4), Al_2O_3 (0.1), SiO_2 (0.3), P (tr), Fe_2O_3 (0.6), Nb (tr)
Ink 6	32.8 ± 0.5	TiO_2 (99.7), P (tr)
Ink 7	45.8 ± 1.3	TiO_2 (99.8), P (tr)
Ink 8	46.8 ± 0.1	TiO_2 (95.7), Al_2O_3 (4.2)
Ink 9	67.8 ± 1.9	TiO_2 (85.9), Al_2O_3 (3.8), Fe_2O_3 (10.2)
Ink 10	37.8 ± 2.7	TiO_2 (95.3), Al_2O_3 (4.6)

* Pigment content was determined by gravimetric analysis. Entries are average ± S.D (n=4).
[a] The elemental composition of pigments was determined by X-ray fluorescence.
[b] Trace (less than 0.1%).

nucleic acids, leading to photocytotoxicity (32–36). The crystalline form of TiO_2 affects its efficiency for generating ROS. Studies have demonstrated that TiO_2 (anatase) is dramatically more photocatalytically active and photocytotoxic than the TiO_2 (rutile) (24,33,37–39). We used X-ray diffraction to determine the crystalline phase of TiO_2 in samples of TiO_2 sold as anatase or rutile and in pigments isolated from permanent makeup inks (Table II). The sample of TiO_2 sold as anatase was predominately anatase but contained 1.8 % TiO_2 (rutile). One of the samples sold as TiO_2 (rutile) contained 4.7% TiO_2 (anatase), while no anatase was detectable in the other sample of TiO_2 (rutile). Anatase was the primary crystalline form of TiO_2 found in the pigments isolated from six of the permanent makeup inks (inks 1, 2, 3, 6, 7, and 10). Ink 5 contained comparable amounts of TiO_2 (anatase) and TiO_2 (rutile). Ink 4, though predominately TiO_2 (rutile), contained 0.6% TiO_2 (anatase). The remaining two inks (inks 8 and 9) contained entirely TiO_2 (rutile).

CYTOTOXICITY AND PHOTOCYTOTOXICITY OF PERMANENT MAKEUP INKS AND PIGMENTS ISOLATED FROM PERMANENT MAKEUP INKS

The described *in vitro* assay allows measurement of the dose-dependent toxicity of permanent makeup inks and the pigments contained in these inks. Figures 2 and 3 depict representative dose-response curves. Figure 2 shows the dependence of survival on the incident dose of light for fibroblasts treated with commercially available TiO_2 (anatase).

Table II
Crystalline Phase of Titanium Dioxide and Light-Induced Effects

Sample	Anatase* (w/w %)	PD_{50}[a] of ink ($\mu g/cm^2$)	PD_{50} of pigment ($\mu g/cm^2$)	ESR relative intensity[b]
Anatase	98.2 ± 0.4	—	0.83 ± 0.14	100
Rutile 1	0	—	>150	27
Rutile 2	4.7 ± 0.1	—	1.20 ± 0.18	21
Ink 1	97.9 ± 0.2	1.73 ± 0.10[c]	1.41 ± 0.18	69
Ink 2	98.5 ± 0.3	3.18 ± 0.28[c]	2.12 ± 0.23	24
Ink 3	97.7 ± 0.5	2.08 ± 0.19	2.97 ± 0.45	65
Ink 4	0.6 ± 0.1	2.44 ± 0.15[c]	1.23 ± 0.10	19
Ink 5	54.7 ± 0.3	2.74 ± 0.23	2.93 ± 0.16	106
Ink 6	100	0.73 ± 0.09	1.03 ± 0.11	156
Ink 7	99.1 ± 0.4	1.74 ± 0.35	1.10 ± 0.18	100
Ink 8	0	>150	>150	ND[d]
Ink 9	0	>150	>150	2
Ink 10	97.1 ± 0.3	2.44 ± 0.08	2.26 ± 0.14	ND

* The crystalline form of TiO_2 was determined by X-ray diffraction. The percentage in the TiO_2 (anatase) form is given. The remaining TiO_2 was TiO_2 (rutile).

[a] PD_{50} is the level of exposure to pigment that, in combination with light, results in a 50% survival measured as colony formation. The PD_{50} ± standard error was determined by fitting data to a four-parameter logistic function using SigmaPlot 8.

[b] Peak-to-peak intensity observed for the second line in the ESR spectrum for the DMPO-OH spin adduct. Intensities are expressed relative to the intensity observed for TiO_2 (anatase).

[c] The PD_{50} determined for this ink is significantly different from the PD_{50} determined for the pigment isolated from this ink ($p < 0.05$).

[d] Not detected.

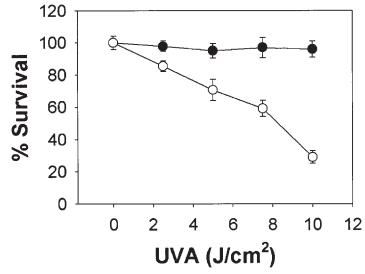

Figure 2. UVA dependence for photocytotoxicity elicited by TiO_2. Human dermal fibroblasts were incubated 18 hours with medium containing 2 $\mu g/cm^2$ TiO_2 (anatase) (open circles). Control samples received no TiO_2 (closed circles). Fibroblasts were then irradiated with a combination of UVA radiation and visible light. Survival was assessed by determining the ability of fibroblasts to form colonies following irradiation. Data points represent the average ± standard deviation of four replicates.

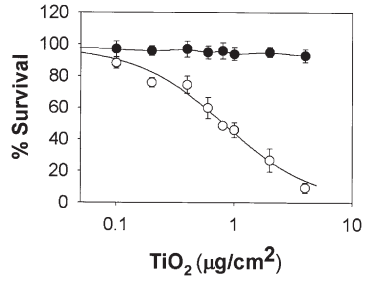

Figure 3. Concentration dependence for photocytotoxicity elicited by TiO_2. Human dermal fibroblasts were incubated 18 hours with medium containing the indicated levels of TiO_2 (anatase). Fibroblasts were then irradiated with a combination of 10 J/cm^2 UVA radiation and 45 J/cm^2 visible light (open circles). Control groups were treated with only TiO_2 (anatase) (closed circles). Survival was assessed by determining the ability of fibroblasts to form colonies following irradiation. Data points represent the average ± standard deviation of four replicates.

Exposure to light alone did not significantly reduce the survival of fibroblasts. Exposure to TiO_2 (anatase) prior to irradiation resulted in a reduction of survival that was dependent on the dose of light. The relationship between level of exposure to TiO_2 (anatase) and survival is shown in Figure 3. TiO_2 (anatase) was not cytotoxic, i.e., no toxicity was seen when fibroblasts were exposed to TiO_2 (anatase) alone. However, TiO_2 (anatase) was photocytotoxic. Exposure to both TiO_2 (anatase) and a combination of 10 J/cm^2 UVA radiation and 45 J/cm^2 visible light resulted in a reduction in survival that was dependent on the level of exposure to TiO_2 (anatase). Using the data shown in Figure 3, the PD_{50} for TiO_2 (anatase) was found to be 0.82 ± 0.14 $\mu g/cm^2$ (Table II). The photocytotoxicity of two samples of commercially available TiO_2 (rutile) was similarly examined. One sample of TiO_2 (rutile), found by X-ray diffraction to contain entirely the rutile crystalline form, did not elicit photocytotoxicity (Table II). The second sample of TiO_2 (rutile), which contained 4.7% TiO_2 (anatase), was photocytotoxic ($PD_{50} = 1.2 \pm 0.18$; Table II). These results demonstrate that TiO_2 containing only a small proportion of the anatase crystalline form can be significantly photocytotoxic.

The results of the photocytotoxicity tests for ten permanent makeup inks are shown in Table II. None of the inks were cytotoxic, i.e., the survival of fibroblasts was unaffected

by treatment with the inks alone (data not shown). However, eight inks were found to be photocytotoxic. Six of the photocytotoxic inks (inks 1, 2, 3, 6, 7, and 10) contained predominately TiO_2 (anatase). Ink 5 contained comparable amounts of TiO_2 (anatase) and TiO_2 (rutile). These results indicate that the photocytotoxicity of the inks is attributable to TiO_2 (anatase). Ink 4, which contained only 0.6 % TiO_2 (anatase), was also determined to be phototoxic. However, ink 4 contained additional pigments as evident from its color (flesh-colored). These additional, unidentified pigments may have contributed to the photocytotoxicity elicited by ink 4. The remaining inks, which contained only TiO_2 (rutile) (inks 8 and 9), were not photocytotoxic.

Inks used for permanent makeup are complex mixtures, generally containing mixtures of pigments dispersed in a multicomponent diluent (1). While it is plausible that the pigments contained in inks elicited the photocytotoxicity we observed, it is possible that components in the diluent may have enhanced or moderated the photocytotoxic response. To investigate this possibility, pigments were isolated from the inks and tested independently using the described *in vitro* method for assessing cytotoxicity and photocytotoxicity. The results are presented in Table II. No cytotoxicity was noted following treatment with pigments alone (data not shown). However, every pigment isolated from a photocytotoxic ink was also photocytotoxic. In addition, pigments isolated from inks that were not photocytotoxic were also not photocytotoxic. These results indicate that the observed photocytotoxicity is attributable primarily to the pigments contained in the inks. For inks 1, 2, and 4, the PD_{50} determined for the ink was significantly larger than the PD_{50} determined for the pigments isolated from the inks (Table II). This result suggests that these inks contain components that can moderate the photocytotoxicity of the pigments. We are currently developing methods to fractionate permanent makeup inks to determine how interactions between components of an ink affect the ink's photocytotoxicity.

In addition to crystalline form, particle size and surface coating can moderate the photocatalytic activity of TiO_2. The reactions photocatalyzed by TiO_2, and resulting in the formation of ROS, occur at the particle's surface. Because the surface-area-to-mass ratio decreases as particle size increases, TiO_2 composed of large particles has diminished photocatalytic catalytic activity due to the relatively lower surface area. In addition, application of a surface coating is a common strategy for moderating the photocatalytic activity of TiO_2 (24). Frequently used surface coatings include inorganic oxides (e.g., Al_2O_3 and SiO_2), dimethicone, and stearic acid (40,41). Analysis by X-ray fluorescence suggests that some of the inks (inks 2, 4, 8, 9, and 10) are formulated using TiO_2 coated with Al_2O_3 or a combination of Al_2O_3 and SiO_2 (Table I). However, several of these inks (inks 2, 4, and 10) were found to be photocytotoxic (Table II). These results are consistent with reports that coating TiO_2, particularly the anatase crystalline form, with inorganic oxides provides incomplete protection against photocatalysis and photocytotoxicity (36,40,42).

GENERATION OF THE HYDROXYL RADICAL FOLLOWING PHOTOEXCITTION OF PIGMENTS ISOLATED FROM PERMANENT MAKEUP INKS

Investigators have shown that ESR is a useful method for detecting ROS formed during photoexcitation of TiO_2. The mechanisms underlying the photocatalytic activity, and particularly the photocytotoxicity, of TiO_2 are not well understood. However, available studies indicate that the hydroxyl radical is the primary ROS responsible for the chemical and biochemical events initiated by photoexcited TiO_2 (31,43). Therefore, we have measured the formation of the hydroxyl radical to characterize the ROS generated during

photoexcitation of pigments isolated from permanent makeup inks. Figure 4 shows the effect of irradiation time on the ESR signal for a suspension containing the pigment isolated from ink 6, which was shown by X-ray diffraction to contain 100% TiO_2 (anatase) (Table II). A four-line ESR signal, characteristic of the spin adduct between DMPO and the hydroxyl radical (DMPO-OH), was observed. The signal intensity increased with increasing irradiation times up to 20 minutes (Figure 4). Figure 5 shows the relationship between the concentration of the pigment from ink 6 and the intensity of the ESR signal, measured as the peak-to-peak height of the second line in the ESR spectrum for DMPO-OH. All samples were irradiated at 320 nm. The intensity of the ESR signal increased for suspensions containing up to 50 μg/ml of pigment and began to plateau thereafter. These results established the appropriate irradiation time (20 min) and concentration (50 μg/ml) for measuring efficiencies of hydroxyl radical generation.

The efficiencies for hydroxyl radical generation, evaluated as the ESR intensity of the sample relative to the ESR intensity of commercially available TiO_2 (anatase), are shown in Table II. Except for the pigment isolated from ink 10, all pigments containing TiO_2 (anatase) photocatalyzed the formation of hydroxyl radicals. There did not appear to be a correlation between the percentage of TiO_2 (anatase) and the efficiency of hydroxyl radical formation for pigments isolated from inks. This suggests that other properties, such as surface coating and particle size, significantly influence the efficiency of these pigments to photocatalyze the formation of hydroxyl radicals. Similarly, except for the pigment isolated from ink 10, all pigments that elicited photocytotoxicity also photocatalyzed the formation of hydroxyl radicals. These results are consistent with the role of ROS in the photocytotoxicity elicited by TiO_2. However, there was little correlation between the PD_{50} measured for a pigment and its efficiency for photocatalyzing the formation of the

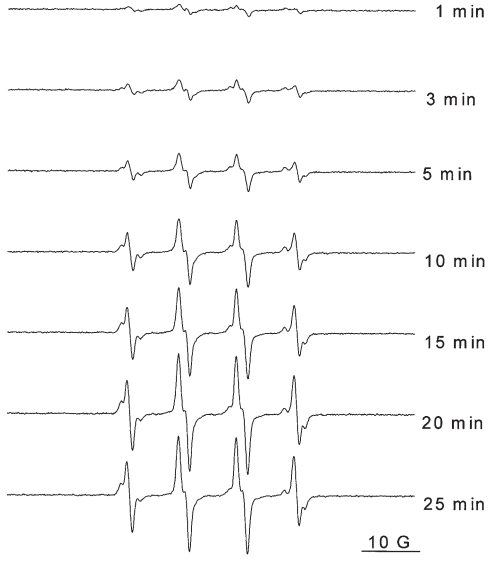

Figure 4. UVA dependence for the formation of the adduct between the hydroxyl radical and the spin trap, DMPO. The pigment isolated from ink 6 (50 μg/ml), suspended in water containing 50 mM DMPO, was irradiated for the indicated times with UV radiation (320 nm). The spin adduct, DMPO-OH, was detected by ESR as described in the Experimental section.

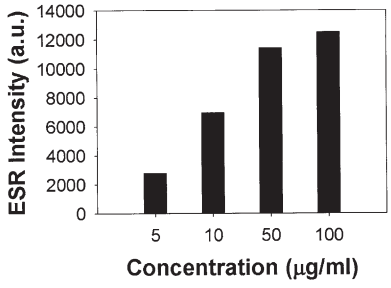

Figure 5. Concentration dependence for the formation of the adduct between the hydroxyl radical and the spin trap, DMPO. The pigment isolated from ink 6 was suspended in water containing 50 mM DMPO and irradiated for 20 minutes with UV radiation (320 nm). For each concentration of pigment, the ESR intensity, in arbitrary units, was obtained by measuring the peak-to-peak height of the second line of the ESR spectrum for the spin adduct, DMPO-OH. The conditions for detection of the ESR signal are described in the Experimental section.

hydroxyl radical. These results indicate that ESR is a useful method for qualitative screening of inks and pigments containing TiO_2 for photocytotoxicity, but that it has limited value for predicting the relative photocytotoxic potentials of these pigments.

CONCLUSIONS

Although there has been a dramatic increase in the popularity of permanent makeup, there has been little progress in the development of toxicological methods to determine the safety of the inks used. Undoubtedly, no single *in vitro* test will be fully adequate to demonstrate the safety of a permanent makeup ink. A battery of tests may be needed to assess the toxic, phototoxic, immunogenic, and carcinogenic potential of these inks. The assay described here allows an *in vitro* assessment of both toxicity and phototoxicity. We have found that this assay is applicable for testing inks used for decorative tattoos as well as permanent makeup (44).

Because of the small number of permanent makeup inks studied in this work, we cannot view our results as a general survey of permanent makeup inks. However, our results suggest that inks containing TiO_2 are frequently formulated with the photocatalytically active and photocytotoxic crystalline form of TiO_2. The clinical consequences of this photocytotoxicity are unclear. Sunlight-induced adverse reactions in skin bearing decorative tattoos and permanent makeup have been reported. In most cases, the pigment(s) responsible for these adverse reactions is not known. However, because TiO_2 strongly absorbs in the UV region of the terrestrial solar spectrum, is widely used in inks, and can elicit *in vitro* phototoxicity, concern over TiO_2-induced phototoxicity is warranted. Another concern is the effect of photocatalytically active TiO_2 on the removal of tattoos and permanent makeup. Investigators have reported difficulties in removing tattoos and permanent makeup using laser ablation. It has been noted that the use of inks containing TiO_2 is associated with some of these adverse outcomes (21,22,45). The use of inks containing photocatalytically active TiO_2 may introduce deleterious photochemical reactions in the skin during laser ablation. An additional issue for individuals obtaining tattoos or permanent makeup is sun-induced fading. Individuals acquiring a new tattoo are commonly instructed to protect tattooed skin from the sun. This precaution is consistent with studies that demonstrate that organic pigments commonly used in tattoo and permanent

makeup inks decompose when exposed to light (46,47). The use of photocatalytically active TiO_2 may further accelerate sun-induced fading. The *in vitro* toxicological method described here should be useful both to manufacturers formulating inks and organizations charged with overseeing the safety of these inks.

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