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Black tattoo inks induce reactive oxygen species production correlating with aggregation of pigment nanoparticles and product brand but not with the polycyclic aromatic hydrocarbon content

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Abstract: Black tattoo inks are composed of carbon nanoparticles, additives and water and may contain polycyclic aromatic hydrocarbons (PAHs). We aimed to clarify whether reactive oxygen species (ROS) induced by black inks *in vitro* is related to pigment chemistry, physico-chemical properties of the ink particles and the content of chemical additives and contaminants including PAHs. The study included nine brands of tattoo inks of six colours each (black, red, yellow, blue, green and white) and two additional black inks of different brands ($n = 56$). The ROS formation potential was determined by the dichlorofluorescein (DCFH) assay. A semiquantitative method was developed for screening extractable organic compounds in tattoo ink based on gas chromatography–mass spectrometry (GC-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Two black inks produced high amounts of ROS. Peroxyl radicals accounted for up to 72% of the free radicals generated, whereas hydroxyl radicals and H_2O_2 accounted for <14% and 16%, respectively. The same two inks aggregated strongly in water in contrast to the other black inks. They did not exhibit any shared pattern in PAHs and other organic substances. Aggregation was exclusively shared by all ink colours belonging to the same two brands. Ten of 11 black inks had PAH concentrations exceeding the European Council's recommended level, and all 11 exceeded the recommended level for benzo(a)pyrene. It is a new finding that aggregation of tattoo pigment particles correlates with ROS production and brand, independently of chemical composition including PAHs. ROS is hypothesized to be implicated in minor clinical symptoms.

Key words: black ink – particle size – polycyclic aromatic hydrocarbons – reactive oxygen species – tattoo

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Introduction

We recently found a high prevalence of complaints in individuals with tattoos; that is, 27% reported minor symptoms such as swelling and itching in tattoos (1). Eighty-five per cent of complaints were confined to black tattoos; however, black tattoos in the same individual might be reactive or not. Fifty-eight per cent of complaints were induced by exposure to sunlight. In contrast to complaints and minor symptoms, medical complications are few or exceptional and mainly reported in red tattoos (2,3). Linking tattoo problems to specific brands of inks is complicated because few tattooed individuals know the brand of the ink installed in their skin.

We have previously measured the size distribution of pigment particles in 58 tattoo inks including 11 black inks and found black inks to contain nanoparticles of amorphous carbon, that is, carbon black (4). Carbon black particles were smaller than the pigment particles of other colours such as red, yellow, blue, green and white (4).

Studies exploring the effects of carbon black nanoparticles *in vitro* (lung cell lines) and *in vivo* (animals) have demonstrated that such particles induce inflammation, oxidize DNA, cause DNA strand breaks and increase the mutant frequency following long-term exposure at a subcytotoxic concentration (5,6). Damage from carbon black has been suggested to be a result of formation of reactive oxygen species (ROS) (7). ROS are reactive molecules that can react with cellular macromolecules, such as lipids, proteins and/or DNA, and compromise normal cellular functions (5,8). Recently, there have been several reports supporting the participation of ROS in the pathogenesis of contact dermatitis. Studies have shown that ROS are generated in dendritic cells, and various chemicals have been shown to produce ROS in a human keratinocyte cell line (9).

Carbon black is produced by controlled combustion. Borm *et al.* (10) showed that various commercially available carbon black pigments differed widely in their polycyclic aromatic hydrocarbon (PAH) content ranging from 0.123 to 329.7 ng/g. Regensburger *et al.* (11) recently found high amounts of PAHs and phenol in black tattoo inks. In addition, they showed that some PAHs produce singlet oxygen on light exposure and that this may explain the decrease in mitochondrial activity in human keratinocytes *in vitro*. However, commercial tattoo inks and their ability to produce ROS without light exposure were not studied in the past. Other substances than PAHs in inks might as well generate ROS. Additionally, Lehner *et al.* (12) analysed 14 black tattoo ink products and found a range of problematic substances with potential health risks.

We aimed to clarify whether the generation of ROS induced by black inks is related to the physico-chemical properties of the inks, such as aggregation state and content of chemical additives and contaminants including PAHs.

Methods

Tattoo inks

We purchased 56 commercially available tattoo inks. Six colours (black, red, yellow, blue, green and white) from nine brands were bought along with an additional two black inks from different brands. The purchases were guided by a market analysis carried out by the Danish Ministry of the Environment identifying the most commonly used tattoo inks in Denmark (13). Inks were aqueous dispersions, ready-to-use inks with water as the vehicle, except one, a paste. The manufacturers of the black inks are named in footnote to Table 1. The purchased coloured inks including the chemical structure of pigments have been specified previously (4). Briefly, the blue and green pigments were phthalocyanines, the yellow and red pigments were azo dyes, whereas the white pigments consisted of titanium dioxide. However, the labelled composition of the inks has previously been published as being widely inadequate (14). Glycerine, isopropanol and witch-hazel are examples of chemicals and additives named on the label of the inks in unknown concentrations. In several cases, the presence of additives was not specified at all and could not be ruled out.

Experiments were conducted on the black inks exclusively apart from measurements of the ROS formation potential, which were performed on all 56 inks. The purpose was to assess constituents versus brands.

Aggregate particle size of black tattoo inks

The hydrodynamic particle size distribution of the black inks was analysed by photon correlation spectroscopy using a Dynamic Light Scattering (DLS) Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK) as previously described (6). The system makes it possible to size the hydrodynamic diameter of particles between 0.6 and 8500 nm. Distributions were calculated by Dispersion Technology Software (Malvern Instruments Ltd., Worcestershire, UK) using the viscosity for H_2O and the refractive index and absorption index for carbon black (2.02 and 2, respectively). Particle suspensions were analysed unfiltered and in the event of analytical difficulties caused by severe agglomeration also following filtration (3.1-, 1.5- and 0.2-μm filters). Five measurements each consisting of 12–16 scans were performed for each filtration. Samples were measured in disposable polystyrene cuvettes containing a 1-ml sample.

Table 1. Particle size obtained by DLS and dry weight percentage of the black tattoo inks

Brand	Mean aggregate diameter (nm)	Dry weight percentages (%)
True Black, Intenze (1)	54[1]	62
BLK, Dynamic Color (2)	55[2]	40
Bad Ass Black, Gold (3)	70	31
Lining Black, Eternal Ink (4)	71[3]	59
Pelikan Ink (5)	84[4]	33
Talens Black Ink (6)	85	39
Tribal Black, Micky Sharpz (7)	90	67
Black Magic, Wefa Color (8)	98	46
Tribal Black, Starbrite (9)	115	59
Midnite Black, Huck Spaulding (MBHS) (10)	ND (>8.5 μm)[5]	33
Real Black Concentrate, National Tattoo (RBNT) (11)	ND (>8.5 μm)[6]	60

ND, not determined; DLS, dynamic light scattering; SEM, scanning electron microscopy.
The products (MBHS and RBNT) agglomerated beyond the range of the DLS, causing unreliable data.
The primary particle size was estimated from SEM images in selected inks; [1]16–43 nm, [2]15–40 nm, [3]12–35 nm, [4]16–40 nm, [5]20–45 nm and [6]20–62 nm.
Number in brackets indicates manufacturer: (1) South Rochelle Park, NJ, USA; (2) Ft. Lauderdale, FL USA (Full name: BLK, Dynamic Color Co.); (3) Moreton, UK; (4) Brighton, MI, USA; (5) Hannover, Germany; (6) Apeldoorn, Holland; (7) Birmingham, UK (Full name: Tribal Black, Mickey Sharpz, Easyflow); (8) Lahnstein, Germany; (9) Ft Lauderdale, FL, USA; (10) Voorheesville, NY, USA (Full name: Midnite Black, Huck Spaulding Enterprises, Inc., VooDoo); (11) Allentown, PA, USA (Full name: Real Black Concentrate, National Tattoo Supply, homogenized tattoo ink).

The water dispersibility properties of the 11 black inks were observed macroscopically by dispersing them in Milli Q water. The dispersibility properties of the remaining 45 inks of other colours have previously been described (4).

Dry weight of black tattoo inks

The dry weight percentages of pigments in the black suspensions were determined by ultracentrifugation [(Optima L-80 XP Ultracentrifuge; Beckman Coulter Inc., Fullerton, CA, USA) at 100 000 g for 2 h]. After ultracentrifugation, we obtained nearly dry powder of carbon black upon removal of the supernatant.

ROS-generating ability of tattoo inks

We measured the ROS-producing ability in nine successive dilutions of the tattoo inks in Milli Q water. The experiments were performed in the dark because the probe was sensitive to light. Two separate experiments each containing their own replicate were conducted with the mean (±SD) being the result. The suspensions were diluted from 400 to 102 400 times corresponding concentrations from maximum 1862.5 μg/ml to minimum 2.71 μg/ml. The ROS-generating ability was assessed using 2′,7′,-dichlorodihydrofluorescein diacetate (DCFH$_2$-DA) as described previously (15). Briefly, 500 μl of 1 mM DCFH$_2$-DA was chemically hydrolysed for 30 min to 2′,7′,-dichlorodihydrofluorescein (DCFH$_2$) by adding 2 ml 0.01 M NaOH. The resulting DCFH$_2$ was diluted to 0.04 mM by adding 10 ml of a 25 mM phosphate buffer at pH 7.4. ROS production by the tattoo inks was determined in Hank's balanced saline solution (without phenol) using a final DCFH$_2$ concentration of 0.01 mM. The formation of 2′,7′,-dichlorofluorescein (DCF) from DCFH$_2$ caused by ROS was determined spectrofluorimetrically following 3 h of incubation in the dark (37°C and 5% CO_2). Excitation and emission wavelengths were λ_{ex} 490 nm and λ_{em} 520 nm, respectively (Victor Wallac-2 1420; PerkinElmer, Skovlunde, Denmark).

The DCFH assay is a general indicator of ROS. To specify which types of ROS were produced by the tattoo inks, experiments were performed with three specific ROS scavengers added to the suspensions of two inks, namely Midnite Black by Huck Spaulding (MBHS) and Real Black Concentrate by National Tattoo (RBNT) (see footnote to Table 1 for details), and to a suspension of pure carbon black (Printex 90; Evonik Degussa, Frankfurt, Germany). Four concentrations of each ink were tested: from 16.23 to 519.38 μg/l for MBHS, from 35.39 to 1132.5 μg/l for RBNT and from 1.88 to 90 μg/l for Printex 90. Mannitol, sodium pyruvate and Trolox were used as a scavenger for hydroxyl radical ($HO^\cdot$), hydrogen peroxide (H_2O_2) and peroxyl radical ($ROO^\cdot$), respectively. The concentration in the final dilutions of sodium pyruvate was 10 mM; mannitol, 20 mM; and Trolox, 100 μM, matching previous recommended levels (16,17).

Extractable organic compounds from black tattoo inks

The ultrasonic extraction procedure was a modification of the method used by Regensburger *et al.* (11), however, carried out as single extractions. Approximately 0.25 ml of each ink sample was weighed accurately in a 1.5-ml polypropylene Eppendorf tube and mixed with 0.75 ml methanol (99.9%, Chromasolv; Fluka, Sigma-Aldrich Denmark ApS, Brøndby, Denmark). It was extracted for 60 min with ultrasonic assistance, left overnight and finally centrifuged at room temperature at 20 000 g for 60 min (Microcentrifuge 157 MP; Ole Dick Instrumentmakers ApS, Hvidovre, Denmark). Blank samples were prepared with the same extraction procedure to test for background contaminants. The supernatant (extract) was used directly for the analysis. Analysis of the more volatile compounds with gas chromatography–mass spectrometry (GC-MS) is described in detail in Data S1 and based on previously described methods (18). Analysis of non-volatile and polymeric compounds with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is described in detail in Data S1 and based on previously described methods (19).

Scanning electron microscopy

Scanning electron microscopy (SEM) images were recorded on a ZEISS EVO MA 10 (Carl Zeiss Ag, Oberkochen, Germany) using 5 kV voltage and 50 pA probe current. The samples were prepared from a suspension of the samples spread on an aluminium disc and allowed to air-dry.

Results

Aggregate particle size of black tattoo inks

The particle size determination by Dynamic Light Scattering (DLS) required a dispersion of the tattoo inks in water. Nine of 11 black inks were fully dispersible in water. The remaining two inks, MBHS and RBNT, were only partly dispersible. The macroscopic aggregates precipitated within a few minutes and the suspensions were therefore not suitable for DLS. Stepwise filtration through 3.1-, 1.5- and 0.2-μm filters was attempted without success. Fig. 1 shows SEM illustrations of the aggregating inks and examples of non-aggregating inks. The primary particle size of MBHS and RBNT was estimated from SEM images and found to be within the nanoparticle range (20–45 nm and 20–62 nm, respectively) and was similar to the other inks, see Table 1. DLS analysis of the nine dispersible inks showed particles with mean aggregate diameters between 54 and 115 nm with a mean aggregate diameter of 80 nm (SD ± 20 nm) (Table 1). The smallest mean diameter was found in True Black by Intenze (54 nm) and the largest in Tribal Black by Starbrite (115 nm). It is commonly agreed that nanoparticles are defined as particles with at least one dimension <100 nm (20). The results of DLS from the present study are in line with the particle sizes measured previously using laser diffraction (Mastersizer 2000; Malvern Instruments Ltd., Worcestershire, UK) (4).

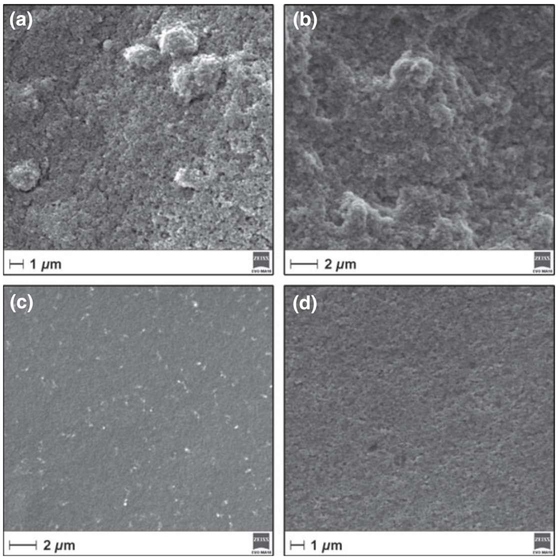

Figure 1. Scanning electron microscopy of two reactive oxygen species (ROS)-producing and aggregating black tattoo inks [(a) Midnite Black, Huck Spaulding, (b) Real Black Concentrate, National Tattoo] and 2 non-ROS-producing and non-aggregating black tattoo inks [(c) Lining Black, Eternal ink, (d) Pelikan Ink].

Dry weight of black tattoo inks

We obtained nearly dry powder after ultracentrifugation of the black inks. The dry weight percentages varied between 31% (Bad Ass Black by Gold) and 67% (Tribal Black by Micky Sharpz) (Table 1). The dry weight percentages of MBHS and RBNT were 33% and 60%, respectively.

ROS-generating ability of tattoo inks

We found large differences in the ROS-producing ability among the 11 black inks analysed. MBHS and RBNT showed a high ROS-producing ability compared with the other black inks, see Fig. 2, and any coloured ink. It is noteworthy that these two black inks were the ones aggregating in water. Furthermore, aggregation in water was shared by inks of all colours (red, yellow, blue, green and white) within the same brand as the two aggregating black inks as previously published (4). The highest generation of ROS was 120-fold above controls observed at a particle concentration of 65 μg/ml (MBHS). The highest ROS production measured by RBNT was 77 times above controls at a concentration of 1133 μg/ml. The declining DCF fluorescence (ROS production) of MBHS observed in Fig. 2 at carbon black concentrations higher than 65 μg/ml has previously been observed and hypothesized attributed to blockage or absorption of the emitted light from the probe at higher particle density (21–23). However, RBNT elicited a continuously increasing response with increasing mass dose. In an attempt to examine the hypothesized particle absorption of emitted light, we centrifuged the samples after the 3 h of incubation and removed the transparent supernatant for reanalysis. Remarkably, the results were similar to the initial setting.

Experiments with specific ROS scavengers revealed that ROO$^{\cdot}$ accounted for 44–69% of the total ROS production (dependent on the tested particle concentration) for MBHS. For RBNT, ROO$^{\cdot}$ accounted for 45–72%. For all tested concentrations, H_2O_2 and HO$^{\cdot}$ accounted for <10% of the total ROS production for MBHS and

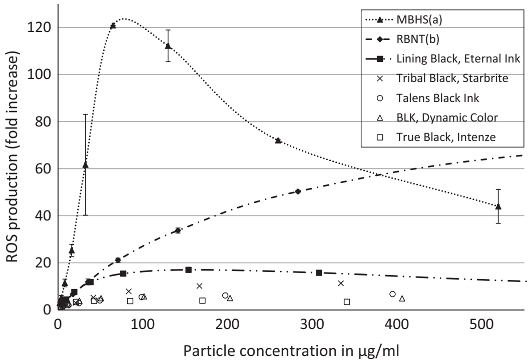

Figure 2. Seven black tattoo inks and reactive oxygen species (ROS)-production in a cell-free solution relative to particle concentration. The remaining four black inks, namely Bad Ass Black (Gold), Tribal Black (Micky Sharpz), Black Magic (Wefa Color) and Pelikan Ink, did not produce ROS and are not illustrated. Standard deviation (SD) is shown for the two ROS-producing inks only. The ROS formation was measured on all 56 inks. The coloured inks generated ROS slightly higher than or similar to background with few exceptions generating ROS more than 10-fold. For details see the Results section. (a) Midnite Black, Huck Spaulding, (b) Real Black Concentrate, National Tattoo.

<16% for RBNT. For the pure carbon black (Printex 90), $ROO^{\cdot}$ accounted for 26–57%, and H_2O_2 and $HO^{\cdot}$ accounted for 1–20% and 0–10%, respectively, depending on tested concentration. Overall, these results are relatively similar to those obtained with the tattoo inks indicating the importance of the pure carbon core. The coloured inks generated ROS only slightly higher than or similar to background with few exceptions generating ROS between 11 and 25 times higher. Exceptions were the products Lightning Yellow (14 times higher) and Graffiti Green (15 times higher) by Eternal Ink, Dark Blue (25 times higher) by Micky Sharpz and Butter Cup Yellow (12 times higher) and Polished Lime Green (11 times higher) by National Tattoo.

Extractable organic compounds from black tattoo inks

The extractable organic compounds from the investigated black tattoo inks showed a wide variety in types and concentrations. Results for PAHs, phthalate esters, phenol, trichlorobenzene and total extractable organic compounds analysed with GC-MS are shown in Table 2. Results for all other tentatively identified compounds are shown in tables in the Data S1. The two ROS-producing black inks are framed in all tables. The extracts of the 11 black tattoo inks were screened for all compounds reported by Regensburger *et al.* and Lehner *et al.*, but we were unable to detect the following seven compounds: hexachlorobutadiene, methenamine, benzophenone, 3,6-dimethyl-1-heptyn-3-ol, 1,6-hexanediol, propylene glycol and 2,2′-oxybis-1-propanol (11,12). All extracted ion chromatograms from m/z = 50 to m/z = 300 were examined for possible compounds in the two ROS-producing tattoo inks. All organic compounds identified with MALDI-TOF-MS were non-ionic surfactants (see the Data S1) and included nonylphenol ethoxylates (Surfonic N-X), octylphenol ethoxylates (Triton X), heptylphenol propoxylates, alkenyl ethoxylates, mixture of polyethylene glycol, isosorbide oligomers and sorbitan ethoxylates (Tween), alcohol ethoxylates and 2,4,7,9-tetramethyl-5-decyne-4,7-diol ethoxylate (Surfynol 4XX). These non-ionic surfactants were not quantified, but are probably present in relatively large amounts. The concentrations estimated by GC-MS are semiquantitative and in equivalents of tridecane. However, authentic standards were used for quantification of PAHs and phthalates. To obtain a full overview of the content of organic compounds in the tattoo inks, the semiquantification also included unidentified compounds resulting in a TIC response in the GC-MS analysis. Table 2 and Tables S1–S3 show that all the investigated tattoo inks consist of 2–3 main components in addition to non-ionic surfactants and relatively high concentrations of PAH. The main components constitute 65–100% of the total extractable organic compounds analysed with GC-MS and are all oxygenated compounds such as butanediol, glycerine and phenol.

Discussion

Two of 11 black tattoo inks deviated by inducing excessive amounts of ROS. Remarkably, the same two inks aggregated in aqueous dispersions in contrast to the remaining nine inks, which were fully dispersible in water. Aggregation was also found among other colours within the same brand and can thus probably be explained by the use of shared additives or by production-related factors (4). The ability to aggregate is presumably dependent on the primary particle characteristics relative to the vehicle and especially physico-chemical surface characteristics including the influences of particle coating and surfactants added. It is a new finding that aggregation of tattoo pigments correlates to ROS production and brand, independently of pigment class and chemical composition. We recognize that ROS production

Table 2. Selected organic compounds extracted from tattoo inks and analysed with GC-MS: PAH, phthalate esters, phenol, trichlorobenzene and total extractable organic compounds.

Compound/property	Lining Black, Eternal Ink	Midnite Black, Huck Spaulding	True Black, Intenze	Tribal Black, Micky Sharpz	BLK, Dynamic Color	Pelikan Ink	Real Black Concentrate, National Tattoo	Tribal Black, Starbrite	Black Magic, Wefa Color	Bad Ass Black, Gold	Talens Black Ink	Blank
PAH (μg/g)												
Phenanthrene	nd	0.22	0.021	0.010	0.006	12.8	0.76	0.45	0.048	0.005	nd	nd
Acenaphthylene	nd	3.6	0.78	0.014	0.13	0.37	0.005	nd	nd	nd	nd	nd
Benzo(b)fluoranthene	0.72	0.080	0.21	0.44	0.73	0.94	0.72	1.99	1.69	1.24	0.073	0.002
Pyrene	0.055	0.39	0.42	0.56	0.65	4.45	0.36	1.07	0.23	0.22	0.11	0.012
Anthracene	nd	0.18	0.025	0.009	0.003	1.26	0.021	0.005	0.002	0.001	nd	nd
Fluoranthene	0.16	1.22	0.89	1.25	0.67	8.19	0.75	0.84	0.45	0.30	0.23	0.027
Chrysene	0.41	0.27	0.025	0.060	0.068	0.23	0.12	0.19	0.29	0.17	0.006	nd
Benzo(a)anthracene	0.13	0.31	0.020	0.069	0.045	0.22	0.11	0.21	0.28	0.18	0.005	nd
Benzo(ghi)perylene	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indeno(1,2,3-cd)pyrene	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Acenaphthene	nd	0.17	nd	nd	nd	0.12	nd	nd	nd	nd	nd	nd
Fluorene	nd	0.006	nd	0.01	nd	nd	nd	nd	nd	nd	nd	nd
Benzo(k)fluoranthene	0.80	0.055	0.10	0.26	0.36	0.47	0.35	1.01	0.92	0.80	0.03	0.003
Benzo(a)pyrene	0.58	0.048	0.086	0.22	0.34	0.37	0.37	1.02	0.90	0.90	0.02	0.001
Naphthalene	0.16	nd	0.039	0.44	0.005	0.034	nd	nd	0.055	nd	nd	nd
Dibenzo(a,h)anthracene	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ΣPAH	**3.00**	**6.59**	**2.61**	**3.33**	**2.99**	**29.4**	**3.56**	**6.78**	**4.86**	**3.81**	**0.46**	**0.04**
Phthalates (μg/g)[1]												
Dibutyl phthalate (DBP)	nd	nd	nd	5.0	nd	1.3	0.7	0.2	3.0	1.2	0.8	0.005
Di-(2-ethylhexyl) phthalate (DEHP)	nd	nd	nd	19.3	0.2	4.2	6.3	0.9	5.2	1.8	3.0	0.006
Phenol (μg/g)	nd	nd	nd	nd	nd	1770	nd	nd	nd	nd	3800	nd
Trichlorobenzene (μg/g)	nd	nd	nd	nd	0.26	0.01	0.72	nd	0.04	nd	0.05	nd
Total extractable organics by GC-MS (μg/g)	**2800**	**7000**	**2600**	**14000**	**1400**	**2900**	**13000**	**600**	**13000**	**800**	**7000**	**200**

NA, not analysed; nd, not detected; GC-MS, gas chromatography–mass spectrometry; PAH, polycyclic aromatic hydrocarbon.

[1] In addition, the extracts were screened for dimethyl phthalate, diethyl phthalate, butyl benzyl phthalate and dioctyl phthalate but none of them were detected.

is highly complex and can be elicited by many different chemical reactions. However, in the present study, the PAHs were not found to play a role under the given experimental conditions (without light).

Peroxyl radicals accounted for up to 69% (MBHS) and 72% (RBNT) of the free radicals involved in the ROS generation. This is higher but still relatively similar to the 56% explained by $ROO^{\cdot}$ in Printex 90. The ROS generation in the two set-ups, particle suspension versus particle free supernatants, showed the same pattern with declining DCF fluorescence upon 65 μg/ml for MBHS. A likely hypothesis is that ROS are produced but react with particle surface or adhered chemicals and not the DCFH probe when particle density reaches a high level. The dissimilarity between MBHS and RBNT may be due to differences in adhered chemicals and their ability to capture ROS.

In accordance with the results obtained by Regensburger *et al.* (11), we found PAHs in all black tattoo inks. We screened for 13 of the 16 U.S. Environmental Protection Agency's (US EPA) reference PAHs, and we found all 13 PAHs in the extracts of the inks (see Table 2).

The Council of Europe recommends less than 0.5 μg/g PAHs (<0.5 ppm) in tattoo inks (24). A PAH content above 0.5 μg/g was demonstrated in 10 of the 11 investigated black tattoo inks. By far, the highest PAH content was demonstrated in Pelikan Ink (29 μg/g), whereas the lowest content, just below the recommendation, was found in Talens Black Ink (0.46 μg/g); paradoxically, this is the only manufacturer of all purchased inks that states that the product should not be used for tattooing. A recent Danish survey on chemical substances in 65 tattoo inks investigated the content of PAHs in black, blue, red, orange and violet tattoo inks (25). Likewise, this study reported a content of PAHs exceeding 0.5 μg/g in 14 of 19 inks, with the highest content of PAHs in the black inks ranging from 0.8 to 117 μg/g.

The most potent PAH detected was benzo(a)pyrene (BaP), which is classified as group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (26). The Council of Europe recommends a maximum concentration of BaP in tattoo inks of 0.005 μg/g (5 ppb) (24). We found BaP concentrations exceeding the recommended level in all 11 analysed samples. The highest concentration was demonstrated in Tribal Black by Starbrite with a value of 1.02 μg/g, that is, 200 times above the recommended level. In comparison, Regensburger *et al.* (11) detected BaP in four of 19 black tattoo inks. Other PAHs of toxicological concern listed as group 2A (probably carcinogenic to humans) or group 2B (possibly carcinogenic to humans) by the IARC were detected such as benz(a)anthracene, benzo(k)fluoranthene, benzo(b)fluoranthene, chrysene and naphthalene (Table 2) (27). There are no separate recommendations on these substances in tattoo ink.

The potential local and systemic carcinogenic effect of tattoos and tattoo inks remains unclear. An updated and thorough review of the literature found 50 cases of cutaneous malignancy in tattoos over the past 40 years (28). Thus, the occurrence of skin cancer in tattoos is surprisingly rare and suggested to be coincidental.

Polycyclic aromatic hydrocarbons are known to increase ROS production leading to oxidative stress (29). The two ROS-producing black tattoo inks did not, however, show any special or similar pattern in PAHs and other organic substances, which could explain the difference in ROS formation. Regensburger *et al.* (11) determined the generation of singlet oxygen from pure PAHs on laser light exposure. They found the highest generation of singlet oxygen from benz(a)anthracene and anthracene. In relation to our study, these two PAHs were found in fairly high concentrations in MBHS but in low concentrations in RBNT (the two ROS-producing inks). The concentration of anthracene was moreover very high in Pelikan Ink, which did not generate ROS. We found no correlation between the total concentration of detected PAHs and ROS. For example, Pelikan Ink with the highest level of PAHs produced less ROS than Talens Black Ink with the lowest level of PAHs. This suggests that other mechanisms besides PAH are active in the generation of ROS. Previous studies have demonstrated that >99% pure carbon black particles (Printex 90) (PAH: 75 ng/g and no adhered metals) were able to produce large amounts of ROS under similar conditions as tested here (15).

It is likely that constituents determining aggregation and ROS production or non-aggregation and no production of ROS are chemical constituents added by the manufacturer, such as emulsifiers, or unknown factors induced by the manufacturing equipment or the method used in the production of the inks. In our study, the pattern of extractable organic compounds could not reveal such chemicals in the inks. It would be practical for a manufacturer to produce one common vehicle for all his products and then add different pigments of different colours to make up his range of product. We cannot, however, cast further light on this issue because the production methods of tattoo inks are not publicly available (14). Interestingly, we found that two inks were similar in their chemical composition and had the same chemical 'fingerprint' (see Tables S1-S3). These were probably the same product sold under different brand names (True Black by Intenze and BLK by Dynamic Color).

Typically, the biological activity of particles increases as the size of the particles decreases (30). Studies on materials such as carbon black show that nanoparticles generate ROS to a greater extent than larger particles of the same material (31). Our results do not support a correlation between particle size and difference in ROS production, but suggest other mechanisms may be important for unpure products such as tattoo inks.

Risk assessments regarding PAHs and nanoparticles have traditionally been based on exposure from inhalation, ingestion and skin contact (32–35). Exposure to coal tar rich in aromatic hydrocarbons may on excessive exposure cause malignancy of the skin, for instance scrotal cancer in chimney sweepers reported back in 1775 (36). However, a large retrospective study of coal tar treatment of psoriasis did not find an association with skin malignancies (32). Nevertheless, tattooing is a common source of PAH and nanoparticle incorporation in the human body with so far unknown risks and consequences. The industrial chemicals added to the inks are often similar to compounds added to waterborne paints such as Texanol, diols and alkoxy ethanols (37). These also may carry risks.

As noted initially, skin complaints and photosensitivity are common in tattooed individuals. However, the causes are often unknown. The total amount of measured additives in the tattoo inks in our study was found in relatively low concentrations of approximately 1% by weight plus the unknown amounts of non-ionic surfactants. These compounds and the added amounts do not appear to be a cause of skin irritation initiated by light. PAHs, on the other hand, are phototoxic and readily absorb sunlight in the ultraviolet range and partially in the visible range (38). The phototoxic action initiated by PAHs is believed to proceed partly via ROS formation (38). We still need to identify a link between clinical symptoms like photosensitivity, ROS production, aggregation of inks and photoactivation of PAH.

The primary route of human phthalate exposure to the general population is presumed to be ingestion (39). We found dibutyl phthalate (DBP) in seven samples and di(2-ethylhexyl) phthalate (DEHP) in eight samples in small amounts (0.2–5.0 μg/g and 0.2–19.3 μg/g, respectively) comparable to the results of Lehner *et al.*(12). Some phthalates such as DEHP and DBP and their metabolites are suspected of being teratogenic and associated with endocrine-disrupting effects (40). The use of DBP in cosmetics, including nail polishes, is banned in the European Union (41).

Trichlorobenzene, a chlorinated aromatic organic compound, was detected in five inks. There is no information available on the health effects in humans (42). The highest value was detected in RBNT (0.72 μg/g).

Phenol was detected in two inks (Pelikan Ink and Indian Ink). Phenol is classified by IARC as group three carcinogen (not classifiable as to its carcinogenicity to humans). However, a number of toxic effects have been described, such as irritation of the skin, eyes and mucous membranes (43).

More researches are needed to explain the outcome of the present study. We evaluated some possible explaining factors such as particle size, PAH and different auxiliaries and found no clear correlation. Future studies might address zeta potentials of particles as well as effect of coating and emulsifiers. The probe we used for ROS detection was sensitive to light, and therefore, the ROS experiments took place in the dark. PAHs may absorb UV light and thereby produce ROS and other reactive intermediates able to damage DNA, proteins and cell membranes (44). These light-induced reactions are another important issue for future study and should be considered as an additive effect. It has previously been shown that PAHs deposited in the skin and UV irradiation may cause skin cancer in mice (44,45). However, clinical reactions to black inks are not exclusively found in sun-exposed skin but also in areas covered by textiles.

Author contributions

The study design, data evaluation, conclusion and reporting were undertaken by the whole team chaired by the first author, who also conducted measurement of ROS production, mass of pigments and particle size by DLS, supported by the second author. Chemical analyses were undertaken by the third author. The fourth author cochaired the study and validated the data and the conclusion and reporting of the study.

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Conflict of interests

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Extractable organic compounds from the tattoo inks.

Table S1. Tentatively identified major compounds, which accounts for a significant fraction of the extractable organic compounds, and which resulted in peaks visible in the TIC trace and with small interference.

Table S2. Tentatively identified minor compounds which are not visible in the TIC trace, however, in concentrations large enough to produce a searchable mass spectrum.

Tabel S3. Organic compounds tentatively identified with MALDI-TOF-MS.