Operable minimum toxicological requirements: 2nd Expert discussion at BfR on tattoo inks

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On 4 May 2022, the second expert meeting on operable minimum toxicological requirements took place at the German Federal Institute for Risk Assessment (BfR) via video conference. Experts from the field of analytics, state surveillance agencies, governmental organizations, tattoo ink manufacturers, and tattooists participated in the meeting.

1 Introduction of the BfR minimum requirements and test methods with emphasis on operable minimum toxicological requirements

The BfR opened the meeting by presenting the definition of the minimum requirements and test methods. It was added that a full risk assessment of tattoo ink pigments is currently not possible since fundamental knowledge gaps exist, e.g. regarding biokinetic distribution in the body and metabolic products after intradermal application. Ingredients of tattoo inks are regulated under REACH. For substances with certain harmonized classifications concentration limits are set for the use in tattoo inks. However, non-classified substances may still be used in tattoo inks. The minimum requirements set up the framework for testing these tattoo ink ingredients according standardized test procedures.

The minimum requirements for tattoo inks aim to achieve two major goals: First, the characterization of physicochemical properties of the ingredients; and second, the minimum toxicological testing of tattoo pigments, which was the topic of the 2nd expert meeting.

It was pointed out that the analytical method development was the topic of the first meeting held on 3 May 2022. The protocol of that meeting is published on the BfR website.[1] Purity and stability during storage as well as exposure to light of tattoo ingredients were points of the discussion during the first meeting.

The toxicological endpoints taken into account for tattoo ink pigments were chosen due to their operability. Hence, systemic effects are not addressed at the moment. The toxicological requirements are set to reduce the possible risk based on the current state of science and technology.

The long term perspective for the risks assessment of tattoo ink pigments is to close knowledge gaps with regard to body distribution and systemic effects. Thus, some short- and medium-term milestones are set to assess the safety of intradermally applied substances.

2 Feedback from participants on the minimum requirements and test methods

[1] https://www.bfr.bund.de/cm/349/necessary-specifications-of-tattoo-ink-ingredients-expert-discussion-at-the-bfr.pdf

The participants acknowledged the comprehensive draft about operable minimum requirements and test methods and its relevance. It is seen as a multidisciplinary approach involving ingredient characterization, toxicological analysis and the envisioned inclusion of epidemiology data from different planned pilot studies with international partners. The participants emphasized the importance of epidemiology taken into consideration since it is estimated that there will be close to 100 million tattooed consumers in the European Union in the near future. Participants acknowledged the importance of including the Absorption, Distribution, Metabolism and Excretion (ADME) parameters, to make the approach more significant. Attendees shared their view on the importance of developing safety assessment guiding protocols like the proposed BfR operable minimum requirements for tattoo ink pigments.

The challenges involved in the risk assessment of these substances were recognized. Manufacturers emphasized that for skin corrosion and skin/eye irritation testing, the concentration limits set up under the REACH regulation need guidance for the manufacturing industry as a common base in the European Union. Tattoo ink manufacturers emphasized further, that the lack of data regarding toxicological endpoints, like phototoxicity/photogenotoxicity, could be a major hurdle. The participants of the ink manufacturing industry expressed their view, that they rely on the existence of a complete risk assessment dossier submitted by pigment producers in the frame of REACH. It was encountered, however, that the current risk assessment of pigments does not include intradermal application. Manufacturers expressed their concern, that only a handful tattoo ink manufacturers are advised by researchers and analytical chemists. However, this would be necessary to make sure that the application is as safe as possible. Furthermore, it was brought up that advice with regard to the preservation of tattoo inks and sanitation measures during the manufacturing process are necessary.

The question was raised, why pigments only are addressed currently in the minimum requirements and test methods. In answer to this, it was stated that pigments were prioritized as the main ingredient of tattoo inks. However, the relevance of a potential toxicity of other ingredients and also mixture effects is acknowledged as well.

Moreover, the question came up, if these kind of requirements are meant to be dynamic and to be applied to pigments available on the market as well as to those entering the market in the future. Concern was raised in regard to the limited information provided from pigment manufacturers. The limits set in the restriction under REACH were addressed. Most limits were set as group limits without a toxicological evaluation. Non-classified substances do not fall into the frame of the restriction. Removal of tattoos is not addressed in the minimum requirements but producers of tattoo inks should consider the fate of the pigments upon irradiation. Participants also raised concerns that interpretation of data from animal studies should be done with caution since there is a huge data gap for intradermal human application. For example, nickel which is ubiquitously used in different consumer products exhibits moderate or low sensitization in animal testing. However, in humans, beside an inflammatory reaction, the sensitizing effect of nickel is enhanced and the element is well known as a sensitizer. Comments were made that the sensitizing properties are not revealed as good as expected in animal testing. Tattoo ink manufacturers brought forward the case of rose oil which is considered a high potential allergen based on epicutaneous testing. It was stated, however, that rose oil in tattoo inks does not cause allergies. Some participants therefore considered the substance not active as an allergen in the dermal layer. Therefore, testing recommendations for intradermal application were requested.

3 Genotoxicity testing of pigments

In the next session BfR introduced the importance of a genotoxicity assessment to identify substances that can cause genetic alterations in somatic and/or germ cells and the importance of using this information in regulatory decisions. Compared to most other types of toxicity, genetic alterations may result in effects that are manifested only after long exposure periods. This endpoint is therefore particularly noticeable for tattoo ink pigment safety assessment. Furthermore, genotoxicity and mutagenicity are at the crossroad of many downstream diseases, such as cardiovascular diseases, immune alteration and neuroendocrinology intervention including endocrine disruption. The BfR minimum requirements and test methods include a battery of genotoxicity/mutagenicity tests to assess the pigment safety following the OECD test guidelines 471, 476, 487 and 490. This includes photogenotoxicity and photomutagenicity. It is worth noting that photogenotoxicity/photomutagenicity testing shall be performed under light exposure in a similar way as genotoxicity/mutagenicity testing. If any of these tests gives a positive outcome, the pigment is unsuitable for use in tattoo inks.

- **Bacterial strains**

BfR introduced the topic of the harmonised OECD test guideline 471 for bacterial mutagenicity testing, also called AMES test, which includes the use of 5 bacterial strains. The modified 5 strain testing with *Salmonella typhimurium* uses genetic modification to make the bacterial membrane permeable. Thus, the uptake of chemicals for identifying their *in vitro* mutagenic potential is facilitated. Furthermore, the test strains are specially constructed to detect either frameshift (e.g., strains TA-1537 and TA-1538) or point (e.g., strain TA-1531) mutations.

The question was raised, whether the mandatory testing with 5 strains as recommended by the OECD should be the part of the operable minimum toxicological requirements. Participants mentioned that if not all 5 strains are tested under REACH, a problem with compliance would exist. In this case ECHA could request a dossier evaluation from the manufacturer. According to the OECD test guideline, AMES tests performed using only 4 bacterial strains only result in incomplete data as shown in the example of Pigment Blue 15:3 mutagenicity assessment as documented at ECHA's public dissemination website.

In view of the aforementioned challenges, participants also discussed to consider alternatives to the AMES test due to concern of false positive results as revealed especially in early AMES studies. In later studies, less false positives were obtained, which supports continued use of the AMES test as a part of the minimum toxicological requirements. Furthermore, the AMES test accuracy may vary from case to case. Considering the diversity of pigments used in tattoo inks, a majority of participants is in consensus that the AMES test should still be used as described by OECD TG 471.

- **Confirmation of uptake**

If substances are not taken up into cells, they cannot exhibit genotoxicity/mutagenicity. Currently, confirmation of uptake is missing in the existing data. This refers to testing with bacterial and mammalian cells. The deficiencies in uptake confirmation are considered as a bottleneck which requires analytical solutions. A consensus was achieved, also due to the nanoparticle nature of tattoo pigments, that cellular uptake must be confirmed using suitable techniques - although it is still not clear which - as outlined in the minimum test requirements.

An *in vitro* toxicologist raised the point that small bacteria of 3-5 µm size are in the same dimension as tattoo pigment agglomerates. This may pose a hurdle to uptake confirmation. Participants reported that in AMES test reporting in the ECHA compliance dossiers, information on both, uptake confirmation and bacterial strains used, is often missing. However, a few reports that confirm uptake were performed with *Saccharomyces cerevisiae* following OECD test guideline 480. All participants agreed, that the uptake needs to be confirmed. High resolution imaging techniques such as transmission electron microscopy or scanning electron microscopy should be considered in that respect.

- **Concentration selection**

Concentration selection and the minimum number of test concentrations are other important points in meeting the acceptability criteria established by the OECD. For all assays, solvent and positive control samples are to be measured in addition to the minimum number of test substance concentrations. The sample size should be adequate to support statistics and data shall be pooled. However, there is no clarity yet regarding the concentration selection. This is particularly important for concentration determination of tattoo ink pigments which are largely insoluble. The sedimentation and agglomeration of insoluble test substances represent major hurdles for concentration determination. The maximum concentration to be chosen is not the one which is mentioned in OECD test guidelines, but it may already vary depending on the selected genotoxicity assay. Accordingly, the test concentration needs to be determined for each individual test rather than relying on OECD recommendations alone.

- **Metabolic activation**

Currently gene mutation testing in bacterial (AMES Test as per OECD TG 471) and mammalian cells is performed with metabolic activation. The rodent liver fraction S9 is used to degrade the test substances into metabolites which could be mutagenic or genotoxic. However, rodent liver fractions are different to human liver fractions and may lead to false positive or negative results. Since the human liver fraction S9 is commercially available, the industry is urged to adopt this for metabolic testing. Challenges with regard to the application of the human liver fraction S9 are seen with regard to fraction homogenization, the need for human donors, and sufficient commercial availability. Participants agreed that being different to human liver S9 fractions, the current research based on metabolic activation using rodent liver fractions could be a concern in genotoxicity assessment because of false negative or positive results. Furthermore, human liver primary cell lines, such as the HepG9 cell line, should be included in pilot studies to identify human liver equivalents for *in vitro* cellular models. This could potentially replace the need for metabolic activation in mammalian gene mutation and micronucleus assays.

4 How to proceed with pigments for which the Ames test is positive and other *in vitro* genotoxicity tests are negative?

Participants agreed that there is clearly a concern for genotoxicity/mutagenicity and that the pigment is unsuitable for use if any of the *in vitro* tests among the proposed battery is positive (including the Ames test). The respective pigments should be banned from usage. The Scientific Committee on Consumer Safety's (SCCS) Notes of Guidance also recommend the justified selection of a battery of validated *in vitro* genotoxicity tests. These could be used for further verification of a positive Ames test in a second tier study. However, the general opinion was that a positive Ames test should result in a ban of the pigment. Participants also suggested that for a substance which is tested positive in an *in vivo* genotoxicity test but negative in *in vitro* tests, the *in vivo* results shall be given precedence and the pigment should not be used in tattoo inks.

- **How to interpret existing *in vivo* data?**

It was discussed to which extent *in vivo* data are more relevant than *in vitro* data. Furthermore, the question was raised if transgenic rodent mutation and rodent comet assays after oral or dermal route applications are suitable or sufficient to replace *in vitro* data. The oral route application for comet assays is generally accepted by regulatory agencies across Europe, Asia and the USA. If the comet assay is used to study the mutagenicity after oral route exposure, the tissue at the site of first entry can be used for the test. A comprehensive compilation of consensus test methods as well as their regulatory and validation status is presented in the SCCS's Notes of Guidance. Overall, a well-founded scientific judgment on available *in vivo* genotoxicity data should be given.

- **Who is in charge of fulfilling or supplying the data required for risk assessment?**

If chemical industry produces more than 1 ton/year of a substance, the supply of data respective to the tonnage produced or imported into the EU becomes mandatory according to the REACH regulation. However, tattoo ink producers may be considered downstream users. Pigment manufacturers do not consider the usage of their products in tattoo inks. Therefore it was discussed who is responsible for fulfilling the requirement for such tests in regard to tattoo ink pigments. However, no consensus was reached. Overall, it was emphasized that if *in vivo* data exist, these should be prioritized for risk assessment.

5 Further views from tattoo ink manufacturers

With regard to the quality of the test reports, tattoo ink producers pointed out that the pigment manufacturers have the responsibility to provide REACH compliance documents on the risk assessment. The tattoo ink producers stated that they do not have or provide their own data but rely on the compliance documents submitted by the pigment manufacturers for substances produced in a range from 1 to 10 tons/year. The ink producers are using a very small amount of the produced bulk pigments. There is no system or regulation in place how to bear the cost of the discussed studies. Tattoo ink manufacturers said that they will not incur such cost since there are many ingredients, which even increase the necessary budget. Other participants pointed out that the U.S. Food and Drug Administration requires 2 *in vitro* and 1 *in vivo* genotoxicity tests for cosmetic products. It was emphasized by tattoo ink manufacturers that overall, decisions to improve tattoo ink safety shall be made on a case by case basis. It

was noted that even if sufficient data for specific pigments are available and revised by regulatory authorities, the assessment of the numerous other tattoo ink ingredients is still needed in order to ensure safer tattoos.

Tattoo ink manufacturers reiterated that they are just a downstream users of the pigments but at the same time the pigment manufacturers are unlikely to shoulder the whole burden. For example, even for one of the most frequently used tattoo pigments, carbon black, the approximate use of about 10000 tons is only a fraction of its yearly production. Participants from the cosmetic industry added that they had been facing a similar situation for materials which have been used in range of 10000 tons/year (or even less for small scale materials, such as certain hair dyes or preservatives) while the manufacturers have not been willing to conduct all the necessary studies.

An approach followed by the cosmetics industry for the assessment of the pigments used in their products is to collaborate/form consortia to fund and conduct the necessary studies. Tattoo ink manufacturers were convinced that their companies will also face similar issues regarding the funding of *in vitro* testing.

Next, further endpoints addressed in the minimum toxicological requirements proposed by the BfR were discussed. A two out of three approach for skin sensitization, as well as the test strategies for skin irritation/skin corrosion and eye irritation/eye damage were discussed.

For pigments which contain metal ions as contaminants, the binding of proteins is a main challenge for skin sensitization testing.

Since pigments are largely insoluble, the reconstructed human corneal epithelium (RhCE) test is questionable since particles are not expected to penetrate intact barriers.

It could be interesting to test tattoo ink pigments with organotypic cultures, e.g. by the Bovine Corneal Opacity and Permeability (BCOP) test or the isolated chicken eye (ICE) test, which could be best suited considering the complex nature of tattoo ink pigments. The Reconstructed Human Cornea-like Epithelium (RHCE) test (OECD 492b) was briefly mentioned with regard to its use for cosmetic pigments. However, participants also admitted that often no *in vivo* data are available for correlation. The participants agreed that the chemical identity and purity of the ink also need to be taken into account when designing *in vitro* tests.

The selection of the right cell model and the comparison between 2D vs. 3D cell models were discussed for phototoxicity testing. 2D cell models for phototoxicity offer advantages regarding the ease of performance, the penetration, the exposure, and the detection since the pigment easily makes contact with the cell monolayer. Even if the test substance is not perfectly soluble, exposure with 2D *in vitro* cell culture systems is feasible. Attention to a potential overexposure may be necessary. However, false positive results caused by the interaction of the cells with the insoluble fraction producing reactive oxygen species (ROS) or membrane damage could pose a major bottleneck.

If a 2D cell model already works fine for phototoxicity testing, there may not be any need to create a complex 3D cell model for which *in vivo* relevance has to be proven. Nevertheless, a balance between sensitivity and specificity must be considered while choosing the 2D vs.

3D cell culture models for risk assessment. The operable minimum toxicological requirements should be flexible in this regard.

Depending on the established *in vitro* methods, the ink manufacturers should use the most appropriate test based on the physicochemical properties of the pigments. In order to reach a consensus with tattoo ink manufacturers for recommending a test, which is rather conservative or overprotecting, the weight of evidence shall be considered. If manufacturers have some benchmark test which reliably detects a positive effect in parallel to an assay drafted in the operable minimum toxicological requirements, such an approach will support and harmonize the joint development of *in vitro* methods.

For *in vitro* phototoxicity testing, the reconstructed Human Epidermis Phototoxicity test (OECD TG 498) including a barrier function could be suitable since it is applied for topically applied substances. However, due to limited migration of intradermally injected tattoo ink pigments, there are also doubts regarding the fit of this test for tattoo ink pigments.

Participants emphasized to adopt more flexibility in the operable minimum toxicological requirements to allow the manufacturers the use of alternative test methods. For eye irritation/damage for example, there should be no limitation to the two proposed assays. Participants stated, that the BCOP test with its short-term exposure is not applicable to solids for the identification of non-irritants.

Moreover, it was questioned why the new test guideline on defined approaches on skin sensitization (OECD 497) is not mentioned in the minimum requirements. Its suitability for the testing of tattoo pigments has to be proven. BfR explained that at the time of publication of the minimum requirements the test guideline was not published. Nevertheless, new methods and guidelines will be considered and included if feasible.

Autofluorescence from the pigments used in tattoo inks and incompatibility of HPLC methods with peptide assays were brought up as further challenges in *in vitro* testing.

Regarding phototoxicity, participants suggested to irradiate the pigments prior to any incubation together with cells. This could be particularly useful for new azo compounds. It would establish an understanding regarding the potential photosensitization by the photodegradation products of irradiated pigments. However, since no validated method and no reliable data exist on this topic, one should proceed cautiously. In developing such a method, the dose response curve, different wavelengths of visible light and the ultraviolet A-B-C spectrum need to be tested.

6 Concluding remarks and next steps

BfR thanked all participants for the fruitful discussion. Furthermore, the progress in the last decade regarding availability of material safety data sheets and information about the supplied ingredients was mentioned. Nevertheless, a further development of tattoo ink risk assessment is considered necessary.

Participants urged to take up the aspect of a size-dependent genotoxicity of TiO_2 pigments. In addition, the importance of choosing suitable cell lines was emphasized because some

might be overly sensitive, especially for genotoxicity assessment of particulate matter. In addition, the intended dynamic dissolution studies with pigments are seen as a suitable approach towards understanding the solubility issue. Furthermore, it was made clear from an epidemiological perspective that photosensitization has to be taken into account as well. Participants were invited to take part in the 3rd expert meeting with focus on toxicological testing requirements to be developed.