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Study Report on Applicability of the key event-based TG 442D for in vitro skin sensitisation testing of nano-materials

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Foreword

This study report on the *"Applicability of the key event based TG 442D for in vitro skin sensitisation testing of nanomaterials"* was prepared by Switzerland, leading the project between 2019 and 2022. Originally it was planned to develop a detailed review paper (DRP). The lead country conducted experimental work with selected nanomaterials using OECD TG 442D (KeratinoSens™) and made a comparison of *in vitro* results to existing *in vivo* information from the literature to outline the potential relevance of the results for any further prediction of skin sensitisation of nanomaterials. A limited number of relevant nanomaterials for testing within this project as well as limited availability of *in vivo* skin sensitisation data for nanomaterials led the lead country, in discussion with experts and the OECD secretariat, to the final conclusion that a study report was the most appropriate way of documenting the outcome of this project and to make it available by publication in the Series on Testing and Assessment for any interested parties carrying out further work related to nanomaterial testing in the area of skin sensitisation.

The study report was endorsed by the Working Party of the National Coordinators of the Test Guidelines Programme at its 35th meeting in April 2023. The study report is published under the responsibility of the OECD Chemicals and Biotechnology Committee.

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1 Executive Summary

Although limited information is available on the *in vivo* skin sensitisation potential of nanomaterials, nine manufactured nanomaterials (MNM) and three positive controls for which some information on their sensitisation potential was available have been identified and subsequently tested according to the KeratinoSens™ test method as described in this study report. Furthermore, transmission electron microscopy (TEM) and dynamic light scattering (DLS) were used to characterise the selected MNM.

Available previously developed standard operation protocols (SOPs) on dispersion and characterisation of MNM were used. The test materials included:

- Two qualities of titanium dioxide nanoparticles and a nickel(II)oxide particle preparation. All three materials showed negative results in the KeratinoSens™ assay. At least partial cytotoxicity at the top concentration indicated that there was cellular exposure.
- Silver and gold were obtained nanoparticles in dispersion form and tested directly without further processing. Both silver and gold gave a clear, dose-dependent luciferase induction in KeratinoSens™ cells. Cytotoxicity was measured in parallel, and positive rating according to the standard prediction model occurred at non-cytotoxic levels.
- Three tattoo inks were tested in addition, as tattoo inks have been discussed in regards to potential induction of skin sensitisation. Both qualities of black tattoo inks were negative in the KeratinoSens™ assay, while a preparation with pigment red 170 tattoo ink gave a clearly positive result at non-cytotoxic concentrations.
- Polyethylenglycoldiacrylate nano-tubes (PEGDA 575) made from organic polymers has been tested negative in KeratinoSens™.

Finally, as a positive control, skin sensitisers (isoeugenol, cinnamic aldehyde and citral) encapsulated in poly-Ɛ-caprolactone particles were tested and compared to the free molecules. In all cases, the encapsulated material gave a similar result as the free sensitisers.

Additionally, a comparison to *in vivo* data from literature was performed, but due to the scarce availability of data it has been done on a qualitative yes/no approach. For PEGDA 575 and the encapsulated poly-Ɛ-caprolactone particles this was not possible as there were no literature data available at all.

As the comparison revealed diverse outcomes for most of the MNM, with available *in vivo* literature (4 were ambiguous and 4 were positive out of the tested 8 MNM), it is not yet possible to draw any further conclusion due to the limited amount of data.

Nevertheless, based on the exchange during the workshops with international experts in the fields of MNM and skin sensitisation, recommendations (e.g., dispersion protocols and viability assay) for an adaptation of the SOP of the KeratinoSens™ test method can be made.

This study showed that technically the KeratinoSens™ test method can be applied for the testing of MNM and the report can serve as a good basis for further work on an adaptation of OECD TG 442D (KeratinoSens™ test method) or related methods.

2 Initial Consideration

Skin sensitisation is a required or recommended end point for testing of chemicals around the world (see Annexe). Besides chemical regulations, skin sensitisation data are also required in several countries in regulations on cosmetics and personal care products, pesticides, pharmaceuticals and medical devices.
Nanomaterials cannot easily penetrate healthy skin. However, studies have shown that nanomaterials can exhibit effects on the immune system even if they do not penetrate the stratum corneum or tight junctions of the epidermis (Hirai et al. 2015). Moreover, in damaged skin, some nanomaterials can pass the stratum corneum and reach the viable skin of the epidermis and dermis (Yoshioka et al. 2017). Besides passage through damaged skin, there are other mechanisms how nanomaterials may be taken up (e.g., penetration through hair follicles or by assisted penetration using delivery systems). After penetration through the skin, different mechanisms may contribute to the immune response. It has been shown that gold nanoparticles can non-covalently bind to skin proteins and thus lead to an immunological response (Premendra D. Dwivedi et al. 2011). Moreover, MNM may, during application, also release unbound chemicals, which might have skin sensitising properties (like metal ions, monomers from not fully cured polymers, small molecules not covalently linked to the nanomaterial matrix). Also, direct uptake of gold nano-particles into immune cells by phagocytosis and pinocytosis has been shown (Dykman und Khlebtsov 2017). While little is known by which mechanism specific nanoparticles trigger allergic reactions *in vivo*, the underlying hypothesis of this project is that nanoparticles deliver and release small chemicals leading to sensitisation. Such effects may be measured with existing tests on skin sensitisation for small molecules.

3 Aim and scope

The aim of this project was to investigate the applicability of OECD TG 442D (KeratinoSens™ test method) for the testing of MNM and to provide, based on the practical experience gained, recommendations for any future adaptations of OECD TG 442D (KeratinoSens™ test method) and nano-related technical improvements to the SOP of the KeratinoSens™ test method.
This project focused on TG 442D (KeratinoSens™ test method) only, but it can be seen as starting point and serve as a basis for future work for adaptation of OECD TG 442D (KeratinoSens™ test method) and for other guidelines and test guidelines related to skin sensitisation testing of nanomaterials. As for other chemicals, any prediction of the skin sensitisation potential of nanomaterials including key event based methods and other data sources will have to be dealt with in the context of Defined Approaches (DA) or Integrated Approaches to Testing and Assessment (IATA). Also, in that sense this project can be seen as an initial contribution and as a basis for any future work.

In this study in particular the following questions were addressed:

- **Is the TG technically applicable to nanomaterials?**
- **Which nanomaterials are suitable for testing?**
- Are there nanomaterials that were not possible to test?
- What has to be adapted to use the TG for nanomaterials?
- Are protocol changes needed to test nanomaterials?

4 Outline of the project

The project has been initially launched within the context of the EU Horizon 2020 project Gov4Nano (https://www.gov4nano.eu/, Grant Agreement number: 814401). The subsequent project at OECD level was taken up to the work plan in 2019 as project 4.133 (lead country Switzerland).

The following work streams were addressed in the margins of this project:

- Selection of MNM to be tested (literature research and first expert workshop Dec 2019)
- Experimental part (technical applicability of KeratinoSens™ test method and characterisation of MNM by TEM and DLS)
- Comparison of *in vitro* results to *in vivo* information from literature to assess the potential of KeratinoSens™ (qualitative correlation to *in vivo* data from literature) to test MNM
- Conclusions/ Recommendations for OECD TG 442D / KeratinoSens™ test method (second expert workshop Dec 2021)

The leads coordinated the work performed by different parties. The work on the KeratinoSens™ has been performed by Givaudan Schweiz AG. The literature review and the comparison of the test results with the available literature information was carried out by TEMAS Solutions GmbH Switzerland (TEMASOL). Characterization of the different MNM has been done by Swiss NanoAnalytics.
According to the work plan two expert workshops have been organised within this project in December 2019 and 2021. Experts from the field of nanotechnology, toxicology, regulation and skin sensitisation have taken part in these workshops. Among them were also experts of the OECD expert group on skin sensitisation. The exchange of information about MNM testing in different skin sensitisation assays and the valuable input by the experts resulted in a selection of MNM for the practical part and recommendations for future adaptation of the OECD TG 442D (KeratinoSens™ test method) and in particular it's SOP.

5 Selection of the MNM to be tested

Manufactured nanomaterials (MNM) were selected following an extensive literature research, exploring studies on toxicological effects of nanomaterials on skin. The critical assessment included data on skin sensitisation of nanomaterials from literature as well as data from all dossiers under the OECD programme for manufactured nanomaterials, where mainly *in vivo* data was retrieved (https://www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm). Identification of relevant literature has been done by systematic research on PubMed and Web of Science with the terms:

- [Nanomaterials] and [skin sensitisation]
- [particles] and [skin sensitisation]

The OECD dossiers from the OECD WPMN Sponsorship Programme for the Testing of Manufactured Nanomaterials (Testing Programme)[1] listed in Table 1 were scanned for potential *in vitro* and *in vivo* data on skin sensitisation.

Table 1 List of OECD Dossiers from Series on Testing on the Safety on MNM

DOSSIER ON CERIUM OXIDE Series on the Safety of Manufactured Nanomaterials no.45 (plus Annexes) (OECD Environment Directorate 2015a)
MULTIWALLED CARBON NANOTUBES (MWCNT): SUMMARY OF THE DOSSIER. Series on the Safety of Manufactured Nanomaterials no.68 (plus Annexes) (OECD Environment Directorate 2016c)
SINGLE WALLED CARBON NANOTUBES (SWCNTs): SUMMARY OF THE DOSSIER. Series on the Safety of Manufactured Nanomaterials no. 70 (plus Annexes) (OECD Environment Directorate 2016e)
DOSSIER ON DENDRIMERS. Series on the Safety of Manufactured Nanomaterials no. 46 (plus Annexes)(OECD Environment Directorate 2015b)
DOSSIER ON NANOCLAYS. Series on the Safety of Manufactured Nanomaterials no. 47 (plus Annexes)(OECD Environment Directorate 2015d)
TITANIUM DIOXIDE: SUMMARY OF THE DOSSIER. Series on the Safety of Manufactured Nanomaterials no. 73 (plus Annexes) (OECD Environment Directorate 2016f)
FULLERENES (C60): SUMMARY OF THE DOSSIER. Series on the Safety of Manufactured Nanomaterials no. 69 (plus Annexes) (OECD Environment Directorate 2016b)
SILICON DIOXIDE: SUMMARY OF THE DOSSIER. Series on the Safety of Manufactured Nanomaterials no. 71 (plus Annexes) (OECD Environment Directorate 2016d)
DOSSIER ON ZINC OXIDE-PART 1, 2 and 3. -Series on the Safety on Manufactured Nanomaterials no 52 (plus Annexes)(OECD Environment Directorate 2015e)
DOSSIER ON GOLD NANOPARTICLES. Series on the Safety of Manufactured Nanomaterials no 44 (plus Annexes)(OECD Environment Directorate 2015c)
DOSSIER ON SILVER NANOPARTICLES-PART 1-7. Series on the Safety of Manufactured Nanomaterials No. 53 (OECD Environment Directorate 2016a)

[1] Testing Programme of Manufactured Nanomaterials - OECD

The OECD Dossiers have been screened for possible MNM to be used in this study.

The review and discussion with experts from the fields of MNM and skin sensitisation during the first expert workshop in December 2019 resulted in a selection of twelve MNM of interest for testing in OECD TG 442D (KeratinoSens™ test method). Table 2 lists the selected MNM that were tested within this project.

Table 2 list of MNM used in the project

MNM name	**Details on identity of the test substance according to reference**	**Skin sensitizing potential and citation**
SG-TO50 (TiO_2)	unknown	Weak sensitizer in OECD Testing Programme (OECD Environment Directorate 2016f)
Titanium(IV)oxide (TiO2)	<25 nm anatase	Auttachoat et al. 2014
Nickel(II)oxide	20 nm SA 40-60 m^2/g spherical	T.R.U.E patch test; due to an allergic reaction to metallic nickel, since no information was found on nickel(II)oxide (Journeay und Goldman 2014)
Gold	Activates Nrf2 pathway	Shows sensitisation potential (Goldstein et al. 2016)
Silver	<10 nm	Kim et al. 2013
Carbon black (Pitch black tattoo ink)	Scream Ink Pitch Black Tribal	Non-sensitizer (Bernatikova et al. 2018)
Carbon black (True Black tattoo ink) (C.I. 7266)	Carbon black	Non-sensitizer (Bernatikova et al. 2018)
Pigment red 170 tattoo ink (C.I. 12475)	4-([4-(Aminocarbonyl)phenyl]azo)-N-(2-ethoxyphenyl)-3-hydroxynaphthalene-2-carboxamide	shows sensitisation potential (Bil et al. 2018)
Polyethylenglycoldiacrylate nanotubes (PEGDA 575)	tube shape	Potential a negative control (Newland et al. 2018)
Isoeugenol	poly-ε-caprolactone nanoparticle (NP) vectors	Used as positive control (Cortial et al. 2015)
Cinnamic aldehyde	poly-ε-caprolactone nanoparticle (NP) vectors	used as positive control (Cortial et al. 2015)
Citral	poly-ε-caprolactone nanoparticle (NP) vectors	Used as positive control (Cortial et al. 2015)

This table lists the MNM selected for this study based on literature research (references given in the last column) and after expert consultation during the first expert workshop.

6 Practical part

The selected MNM to be tested within this study, were analysed with Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) prior to be prepared for application on KeratinoSens™. Sample preparation had to be adapted on MNM and differed to the sample preparation of conventional chemicals.

Sample preparation – Implementation of dispersion protocols

This project benefited from prior international efforts which have already established key testing protocols for nanomaterial safety assessment following *in vitro* strategies. Thus, the SOPs that have been developed within NANoREG framework for the preparation of the different MNM were used within this project[2].

Key publications such as the NANoREG framework and toolbox, which contains a list of recommended protocols focusing on nanomaterial characterisation were carefully reviewed before, during and after experimentation. Evaluation of key parameters such as dissolution, size, agglomeration state both as pristine materials and in relevant media followed published strategies (Dekkers et al. 2016, NanoGenotox, NANoREG) depending on the particulars of the selected materials.

Therefore, the ultra-sonication probe was calibrated according to *"SOP for probe sonicator calibration of delivered acoustic power and de-agglomeration efficiency for ecotoxicological testing; Version 3, January 2015" (NANoREG 2015b)*.

Prior to MNM characterization and exposure of the cells within the KeratinoSens™ the MNM were dispersed and sonicated according to the dispersion protocol of "NANOGENOTOX dispersion protocol for NANoREG" (NANoREG). The main steps of the NANOGENOTOX protocol include a prewetting EtOH step followed by dispersion in 0.05% bovine serum albumin (BSA)-water at a concentration of 2.56 mg/ml using probe sonication.

The characterisation analyses of the test samples had two goals, firstly to assess the initial size distribution and secondly to determine the agglomeration/aggregation after 48 hours in cell culture media, which represents the exposure time according to OECD TG 442D (KeratinoSens™ test method).

Testing in KeratinoSens™: MNM samples delivered as dry powder

Samples were dispersed according to the NANOGENOTOX dispersion protocol from NANoREG at a final concentration of 2.56 mg/mL in 0.05% bovine serum albumin (BSA) solution. This dispersion was diluted 2.5 fold in Dulbecco's Modified Eagle Medium (DMEM) cell culture medium containing 1% fetal calf serum (FCS) (final level of nanomaterial 1024 µg/mL). Eleven 2-fold serial dilutions of this stock dispersion in cell culture medium containing 1% FCS were prepared. The medium from cells grown in 96-well plates overnight according to the SOP was removed and replaced with 150 µL medium containing 1.33% DMSO and 1% FCS. Then 50 µl of the serially diluted stock dispersions were added to the different wells, leading to a final concentration at the top concentration of 256 µg/mL of the test powder and 1% DMSO. With this approach it is ensured that the maximal exposure concentration is equal or above 200 µg/ml, which is the threshold in the gravimetric prediction model of the KeratinoSens™ assay.

[2] NANoREG Results Repository | RIVM

This approach was applied to the titanium dioxide samples, the nickel(II)oxide sample and also to the pigment red 170 tattoo ink.

Testing in KeratinoSens™: MNM samples delivered as dispersions

The dispersion of silver was directly serially diluted in eleven 2-fold serial dilution steps in DMEM cell culture medium containing 1% FCS.
The dispersion of gold was diluted twofold in medium with 1% FCS and then serially diluted in eleven 2-fold serial dilution steps in DMEM cell culture medium containing 1% FCS.
The concentrations tested for these dispersions are directly related to the original dispersion, thus 10,000 ppm indicates 1% of the original dispersion, and concentrations are not normalised to actual content of particles. The medium from cells grown in 96-well plates overnight according to the SOP was removed and replaced with 150 µL medium containing 1.33% DMSO and 1% FCS to assure a final concentration of 1% DMSO.
The carbon black tattoo inks True Black and Pitch Black were diluted to 4 mg/mL in 0.05% BSA solution and then 2.5-fold diluted in DMEM cell culture medium (1.6 mg/mL) and then serially diluted in eleven 2-fold serial dilution steps in cell culture medium containing 1% FCS. The medium from cells grown in 96-well plates overnight according to the SOP was removed and replaced with 150 µL medium containing 1.33% DMSO. Then 50 µL of the serially diluted stock dispersion of the tattoo inks were added to the different wells, leading to a final concentration at the top concentration of 400 µg/mL of the tattoo inks (in terms of tattoo ink suspension, not solid matter) and 1% DMSO.
For the positive controls (caprolactone particles, (Cortial et al. 2015)), which were also delivered as a dispersion (0.52 – 0.66% solid matter based on the load of the test chemical), 2-fold dilutions in the test medium were prepared. These were further diluted 2-fold with test medium containing 8% DMSO. From this master dilution, eleven 2-fold dilutions were prepared. Then 50 µL of these serially diluted stock dispersions were added to the different wells containing the cells and 150 µL of medium, leading to a final concentration at the top concentration of 325 – 412.5 µg/mL based on solid matter of the test chemical and 1% DMSO.
In all these selections of the test range, the maximal concentration was equal or above 200 µg/ml which is the threshold in the gravimetric prediction model of the KeratinoSens™ assay or it is the maximal achievable concentration.
In parallel, control solution with the free positive controls (i.e., cinnamic aldehyde, citral and isoeugenol) and the same nominal load were prepared to obtain equal concentrations of the free and encapsulated test chemicals and the same standard 1% DMSO concentration.

Physical characterisation of manufactured nanomaterials

DLS and TEM are two different techniques that allow to determine the size of the particles but based on different physical principles. DLS and TEM have been chosen for characterization due to prior international projects developing SOPs for these techniques and due to availability of the techniques at the contracting partner for this work. The report from JRC describes in detail the techniques with their limitations (Rauscher et al. 2019).
DLS is an ensemble technique, where the particles are suspended in a solution. When particles are dispersed in a liquid they move randomly in all directions. During the measurement, the incident laser light gets scattered in all directions. The scattered light is detected at a certain angle over time and this signal is used to determine the diffusion coefficient and the particle size by the Stokes-Einstein equation (Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials 2022). The obtained results is the hydrodynamic diameter and there are no differentiation made between the constituent particles and the aggregates (Rauscher et al. 2019).

TEM is an imaging technique that allows to identify the integral components of an agglomerate or aggregate (Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials 2022).
Thus, the obtained diameter from those two techniques may be very different depending on the stability of the powder in the suspension. However, the information from DLS and TEM measurements give an idea of what the test system has been exposed to e.g., how the MNM look like in cell culture media when they were added to the KeratinoSens™.

Transmission Electron Microscopy (TEM)

Initial TEM results indicated aggregations/agglomeration of the MNM samples and polydispersity. Therefore the applied TEM samples preparation technique described in the NANoREG D2.10 SOP 1.0 (NANoREG 2015a) was not optimal. This “grid on the drop” method shows bias towards larger particles and aggregates/agglomerates. In order to obtain a representative TEM sample, a previously described technique was used (Michen et al. 2015) following the drop on a grid approach. MNM samples were prepared by depositing 5 µL of obtained dispersions on a grid (Formvar-coated 200 mesh copper grid) and were imaged the following day.
The imaging setup consisted of a Tecnai Spirit - BioTwin lens | 120 kV LaB6 emitter equipped with a Veleta 2048x2048 camera. To set the optimal parameters for the sample imaging, the NANoREG D2.10 SOP 02 (NANoREG 2015a) was followed TEM images of nanoparticle dispersions were taken at minimum 10 randomized positions on the grid as per NANoREG SOP (NANoREG 2015a). The image analysis the NANoREG D2.10 SOP 03 (NANoREG 2018b), NANoREG D2.10 SOP 04 (NANoREG 2018a) and NANoREG D2.10 SOP 05 (NANoREG) were followed. To analyse the images, the EPFL ELN software, as described in Jablonka et al. 2022, was used.

Dynamic Light Scattering (DLS)

Following the NANOGENOTOX dispersion protocol for NANoREG, samples were diluted equivalent to the highest concentration assessed in the KeratinoSens™ studies. Namely, nickel(II)oxide, the two titanium dioxides, and the pigment red 170 tattoo ink dispersions were diluted down to 0.256 mg/mL, gold and silver were diluted 1:8 and 1:4 fold respectively, while the two carbon black tattoo inks (Pitch Black and True Black) were diluted down to 0.4 mg/mL. Those sample concentrations turned out to be too concentrated for the DLS measurements except the gold and silver samples, for which the stock dispersions were already too diluted.
For that reason, nickel(II)oxide, the two titanium dioxides, and the pigment red 170 tattoo ink were further diluted 10x to 0.0256 mg/mL, while the two carbon black tattoo inks (Pitch Black and True Black), due to the strong interference with the laser light, had to be further diluted 1000x to 0.4 µg/mL. Due to their low concentrations the stock dispersions of gold and silver samples were not diluted any further.
DLS measurements were performed following the NANoREG SOP (NANoREG) as close as that was possible, considering that, the measurements were performed on a Brookhaven Z Sizer with a fixed angle of 90° as opposed to using a recommended Malvern ZetaSizer at an angle of 173°. Furthermore, samples were additionally analysed with a 3D DLS by LS Instruments at angles of 37° and 90°.

Sample stability assessment

Sample stability was assessed in the KeratinoSens™ medium (DMEM containing 1% FBS and 1% DMSO) at 0 and 48 hours incubation at 37 °C. Samples were incubated at the highest concentration used in the cell exposure, but were analysed at the diluted concentrations due to strong interference with the laser light. Except gold and silver, these samples were not further diluted from stock dispersion for analysis with DLS.

KeratinoSens™

The general method is described in the SOP of the KeratinoSens™ assay, published by ECVAM as Dbalm protocol 155 (ECVAM DB-ALM Protocol 2014) and revised in 2017 (155_M_KeratinoSens.pdf (europa.eu)).

Materials

Table 3 and Table 4 list the specifications of the positive controls and the negative/solvent control used. All test reagents were sourced as indicated in the standard operating procedure. Luciferin was sourced from Promega. The Luciferase substrate was prepared according to the following recipe: 20 mM Tricine; 2.67 mM $MgSO_4$; 0.1 mM EDTA; 33.3 mM DTT; 270 µM Coenzyme A; 470 µM Luciferin; 530 µM ATP; pH 7.8.

Table 3 Positive control specification

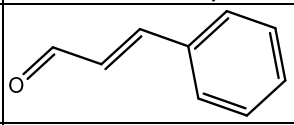
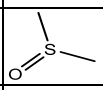
Trivial name	Cinnamic aldehyde
Chemical structure	
Chemical name	Trans-Cinnamaldehyde
Molecular weight	132.16
Molecular formula	C_9H_8O
Purity	>99%
Supplier	Sigma-Aldrich
Product code	239968
CAS number	104-55-2 / 14371-01-9
EC number	203-213-9
Batch number	STBB9109V / 101121271
Physical form	Liquid
DMSO solubility	Freely soluble at 200 mM
Water solubility	1.1 g/L (20 ºC)
Treatment prior to testing	None
Concentrations tested	64 µM, 32 µM, 16 µM, 8 µM, 4 µM
Storage conditions	4°C

Table 4 Negative (vehicle) control specification

Trivial name	Dimethylsulfoxide
Chemical structure	
Molecular weight	78.13
Molecular formula	C_2H_6OS
Purity	>99.5%
Supplier	Sigma - Aldrich

Product code	D5879
CAS number	67-68-5
Batch number	A0385951 / 127791000
EC number	200-664-3
Physical form	liquid
Concentration tested	1%
Storage conditions	Ambient Temperature

Basis of the method

A key common feature for many skin sensitisers is their intrinsic electrophilicity or their potential to be metabolically transformed to electrophilic chemicals. Protein modification can also happen by radical reactions or non-covalent complex formation. The signalling pathway with the repressor protein Keap1 (Kelch-like ECH-associated protein 1) and the transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2), which binds to the antioxidant / electrophile response element (ARE / EpRE), is known to respond to electrophilic chemicals and it was found to be a valuable cellular endpoint to detect skin sensitisers *in vitro* (Natsch und Emter 2008). This result was confirmed by independent laboratories (Ade et al. 2009, Johansson et al. 2011, Miyazawa und Takashima 2012, Ramirez et al. 2014, van der Veen et al. 2013, Vandebriel et al. 2010). Nanoparticles in principle can release both organic and metal allergens, and they can deliver these sensitisers by diffusion and leaching or after being taken up by the cells. In both cases, they may trigger the pathway also activated by dissolved small chemicals.

Test System(s)

The KeratinoSens™ cell line is derived from the human keratinocyte culture HaCaT. It contains a stable insertion of a Luciferase gene under the control of the ARE-element of the gene AKR1C2 (Emter et al. 2010).
The KeratinoSens™ cell line was developed by the testing lab and stored on liquid nitrogen. It was grown in 10 cm petri dishes as described in the SOP to 80% confluence prior to testing for 3 – 4 days.
Cells were counted using a counting chamber and adjusted to the desired density. During seeding into 96-well plates, the cell suspension was gently stirred and cell sedimentation was avoided by repeatedly pipetting up and down to ensure homogeneous distribution of cells. Cells are grown in DMEM supplemented with 9% FCS.

Basic Procedure

Cells are grown for 24h in 96-well plates. The medium is then replaced with medium containing a final level of 1% of the solvent DMSO containing the test substance/nanomaterials. Complex products with no defined molecular weight are tested in the range from 0.2 to 400 µg/mL (which is equal to testing a molecule with MW = 200 at a concentration range from 0.98 to 2000 µM). Each test plate contains six wells with the solvent control, 1 well with no cells for background value and 5 wells with a dose response of the positive

control cinnamic aldehyde. In each repetition, three parallel replicate plates are run with this same set-up, and a fourth parallel plate is prepared for cytotoxicity determination.
Note: While the test range of KeratinoSens™ is up to 400 µg/mL, the prediction model goes up to 200 µg/mL, i.e., a chemical is rated negative if the luciferase induction does not pass 1.5-fold threshold up to 200 µg/mL. This threshold was defined by the testing of the "Silver list" of bona fide sensitisers and non-sensitisers (Emter et al. 2010). While for practical reasons the top concentration was slightly different for materials prepared in different ways in this study, the maximal concentration of the prediction model of 200 µg/mL was reached for all materials rated negative. If a material is rated positive at a lower concentration, the maximal test concentration is not critical.

Positive control

In each test cinnamic aldehyde is included as positive control. It is tested in each test plate at five concentrations from 4 – 64 µM.

Endpoint & Endpoint Detection

Two endpoints are measured: (i) Luciferase induction after a 48h treatment with test substances and (ii) cytotoxicity as determined with the MTT assay recorded in a parallel plate with the same cell batch and made up with the same dilutions of the test substances/nanomaterials.
In the experiments conducted here, a second assessment of viability was performed due to known interference problems with the MTT assay and MNM (Ong et al. 2014). The second viability assay can be performed on the same cells prior to luciferase measurement, this is already described in the revised DB-ALM protocol, but does not form part of the OECD guideline:
Cells were thus grown in white plates with a transparent bottom. At the end of the incubation period, the medium was aspirated and 100 µl of PrestoBlue™ reagent (Invitrogen, Zug, Switzerland) diluted 10-fold in DMEM without phenol red was added to each well

- Plates were incubated for 30 min at 37°C and 5% CO_2.
- The fluorescence at 560 nm excitation and 590 nm emission was determined.
- Cells were rinsed with 125 µL phosphate-buffered saline (PBS).
- Cells were then lysed with 20 µL Passive Lysis Buffer (Promega Duebendorf) at room temperature for 30 min according to the SOP.

Luminescence was read in a Promega Glomax Luminometer programmed to

I. add 50 µl of the luciferase substrate to each well,
II. to then wait for 1 second and
III. then to integrate the luciferase activity for 2 seconds.

Endpoint Value

For Luciferase induction the maximal fold-induction over solvent control (I_{Max}) and the concentration needed to reach a 1.5- induction (EC1.5) are calculated. For cytotoxicity the IC50 value is extrapolated.

Data Processing

Data evaluation is automatically performed by a standardised Excel template, which forms part of the SOP. The test plates are read by a plate reader, and the generated raw data are directly pasted into this template, and all data processing is performed automatically by this Excel sheet.
For both the MTT and the luciferase data, first the background value recorded in an empty well without added cells is subtracted.

For the MTT data the % viability is then calculated for each well in the test plate in relation to the average of the six solvent control wells. The same approach is applied to the PrestoBlue™ assay, with the only difference that for the PrestoBlue™ three replicate plates are available.
For the luciferase data the average value of the six solvent control wells is set to 1, and for each well in the test plate the fold induction is calculated in relation to this value.
The following parameters are then calculated from these processed raw data:

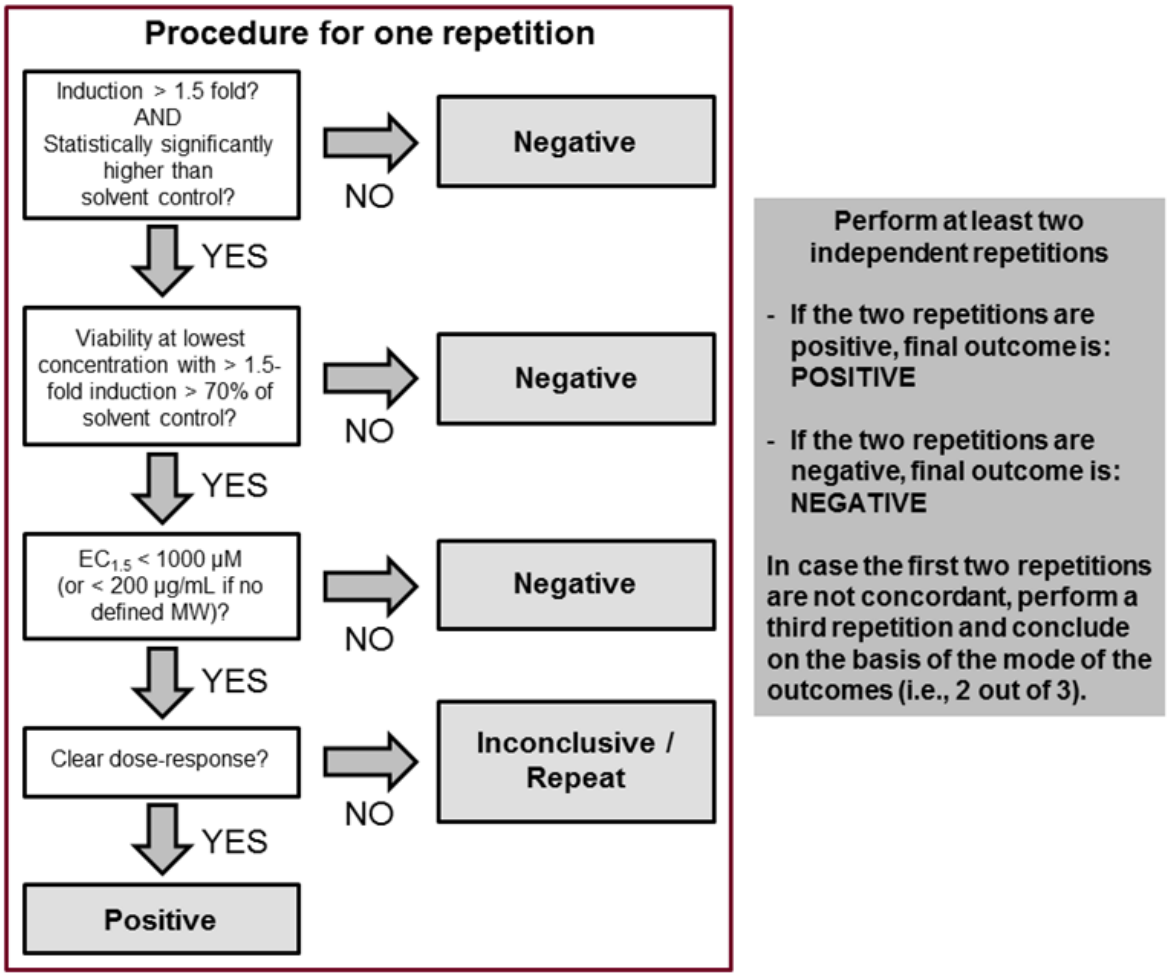
- I_{Max} Maximal fold-gene induction of the luciferase gene over the full dose-response up to 1000 µM (200 µg/mL if no defined MW)
- EC1.5 Concentration in µM for 1.5-fold gene induction (µg/mL if no defined MW)
- Pos/ Neg Rating of substance according to prediction model
- reps. Positive Number of independent repetitions positive / number of repetitions done
- IC50 Concentration in µM for 50% reduction of cell viability (µg/mL if no defined MW)

Prediction Model

Substances are rated positive if the following conditions are met (see also Figure 1):

- The I_{Max} indicates > 1.5-fold gene induction, and this induction is statistically significant above the solvent control in a particular repetition as determined by Student's t-test. The EC1.5 value is below 1000 µM (or 200 µg/mL in case MW is not defined) in all three repetitions or in at least 2 repetitions. (If the I_{Max} is exactly equal to 1.5, the substance is still rated negative and no EC1.5 value is calculated by the evaluation sheet).
- At the lowest concentration with a gene induction above 1.5-fold (i.e., at the EC1.5 determining value), the cellular viability is above 70%.
- There is an apparent overall dose-response for luciferase induction, which is similar between the repetitions.

Figure 1 Prediction model of the KeratinoSens™ assay



Testing of Proficiency chemicals and historical positive control data in the test facility

The KeratinoSens™ assay was originally developed at the testing facility. Data for the proficiency chemicals as defined by OECD TG 442D generated in the laboratory are summarized in the Givaudan report RCR 153'464 'KeratinoSens™ assay: Proficiency testing at the testing facility' (Natsch et al. 2015). The validation of the luciferase readings according to Annexe 3 of the OECD guideline is also given in that report.

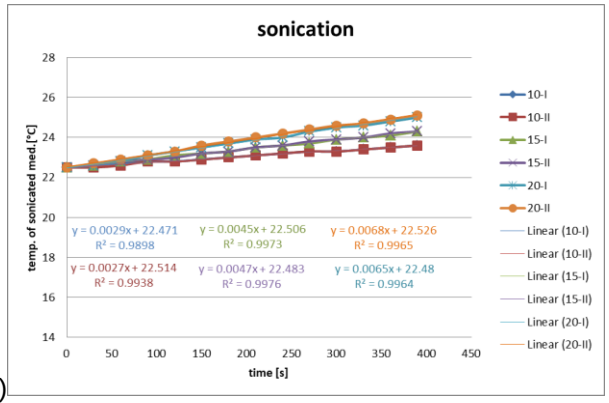
7 Results

Sample preparation – Implementation of dispersion protocols

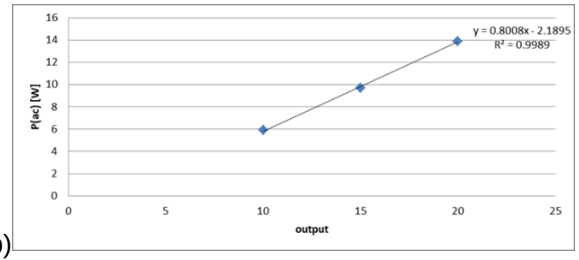
For sonication, the standard Branson sonicator was used with the probe as recommended in the protocol (Branson 101-147-037 Tapped Step Horn with Threaded Body). Based on the measurement of heat generated as per calibration protocol (Figure 2), the energy of 7056 Joule was delivered by 12 min sonication at 15% output in a 6 mL sample in 2.56 mg/mL BSA solution.

Figure 2 Heat generation in the sonication protocol calibration (a) and calibration curve to determine the required % output to deliver the correct required energy of 7056 Joule (b).

a)



b)



Physical characterisation of manufactured nanomaterials

Transmission Electron Microscopy (TEM)

Selected MNM were analysed with TEM. However, Polyethylenglycoldiacrylate nanotubes (PEGDA 575) and the positive controls of citral, cynamic aldehyde and isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors have not been analysed by TEM. The provided amount of polyethylenglycoldiacrylate nanotubes (PEGDA 575) was too little to be used for additional characterisation. Therefore, the value size of polyethylenglycoldiacrylate nanotubes (PEGDA 575) was taken from the respective publication (Newland et al. 2018). And the encapsulated positive controls could not be prepared for TEM imaging without destroying them. The summary of nanoparticle characterisation is given in Table 5 (for more detailed information see also Table 11 in the Annexe).

Significant variability in the data obtained for median, mean and mode of the nanoparticles as well as the broad range of measured sizes indicates that samples are polydisperse and aggregated/agglomerated (Table 5 and Table 11 in the Annexe). The only two samples without signs of aggregation/agglomeration, and a narrow size distribution, were gold and silver nanoparticles. Those two samples are also the only

two with apparent high sphericity. The other samples, likely due to their aggregation/agglomeration, appear as aggregates/agglomerates of various fractal dimensions.

Table 5 Size determination by TEM of the MNM used within this study

Name	Median (nm)	Mean (nm)	SD (nm)
Polyethylenglycoldiacrylate nanotubes (PEGDA 575)*	~ 7'500 (diameter 200nm)	-	-
Nickel(II)oxide	22.5	34.5	35.3
Titanium(IV)oxide	46.8	61.6	51.3
SG-TO50 (TiO_2)	91.4	224.7	142.6
Gold	5.8	360.4	258.1
Silver	6.7	6.0	1.0
Carbon black (True Black tattoo ink)	327.1	7.1	2.2
Carbon black (Pitch Black tattoo ink)	195.3	168.3	172.1
Pigment red 170 tattoo ink	168.2	231.8	249.6

***TEM data of Polyethylenglycoldiacrylate nanotubes (PEGDA 575) were retrieved from Newland et al. (2018)**

Dynamic Light Scattering (DLS)

The summary of DLS measurements is given in Table 6. DLS measurements were performed using two instruments, Brookhaven instrument and LS instruments respectively, for crosschecking results. Since most of the samples appeared to be aggregated/agglomerated, the resulting sizes are estimated based on the intensity-weighted distribution (data not shown).

Gold and silver, even at the stock dispersion concentration, were insufficiently concentrated to generate a sufficient scattering profile, which is evident by the low count numbers. Average size measured for gold and silver were more appropriate with LS instruments, due to the more powerful laser and consequently higher count number. Therefore, these measurements better correlate with the size obtained by TEM. However, gold and silver showed to be insufficiently concentrated to generate a sufficient scattering profile, which is evident by the low count numbers, making the results unreliable.

For the other nanomaterials both instruments indicated similar sizes. Histograms obtained by Brookhaven instrument indicate sample polydispersity (data not shown), which was also detected by TEM measurements.

DLS values (Table 6) of isoeugenol, citral and cynamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors were provided by the producers of these nanomaterials as they had only a short shelf life of one month after production and were therefore measured right on the spot (see in Annexe: Technical information of tested nanomaterials). No DLS values for polyethylenglycoldiacrylate nanotubes (PEGDA 575) were available.

Table 6 DLS sizing - data summary of MNM measured with Brookhaven instrument

Sample	Average size (nm)	Uncertainty (nm)	Polydispersitiy Index (PDI)
Nickel(II)oxide	395	3.1	0.212

Titanium(IV)oxide	233	4	0.224
SG-TO50 (TiO_2)	252.3	2.8	0.186
Gold	57.9	1.9	0.364
Silver	31.5	0.4	0.318
Carbon black (Pitch Black tattoo ink)	221.9	1.1	0.095
Carbon black (True Black tattoo ink)	281.9	1.3	0.121
Pigment red 170 tattoo ink	210.4	1.5	0.128
Isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors*	147.2	41.6	0.054
Cinnamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors*	148.7	39.5	0.028
Citral encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors*	139.6	43.9	0.066

***DLS values of isoeugenol, citral and cynamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) were provided by Cortial et al. 2015 (see also Annexe: Technical information of tested nanomaterials).**

Note: No DLS values for polyethylenglycoldiacrylate nanotubes (PEGDA 575) were available

Sample stability assessment

Sample stability was assessed at 0 and 48 hours. Due to strong interference with laser light, nickel(II)oxide, both titanium dioxides and pigment red 170 tattoo ink were diluted 10x to 0.256 mg/mL, whereas both types of carbon black tattoo inks were diluted 1000x to 0.4 µg/mL. Gold and silver were measured at stock dispersion as these two were low concentrated, which made a further dilution unnecessary.
Because most of the samples appeared to be aggregated/agglomerated, measurements were performed on a Brookhaven Z Sizer with fixed angle of 90° (Table 7) and additionally analysed with a 3D DLS by LS instruments at angles of 37° and 90° (data not shown).
On average, the nanomaterials size increased upon dispersion in cell culture media, which is to be expected due to the likely formation of a protein corona (Balog et al. 2015). Such behaviour in the case of aggregated/agglomerated samples cannot be taken as a rule, since the presence of proteins can lead to the stabilisation of smaller agglomerate fractions as well as influence the measurements by the scattering of the proteins themselves (Balog et al. 2015).
Nickel(II)oxides and both titanium dioxides, showed an apparent decrease in nanoparticle sizes following the 48h incubation, however based on the data given in Table 7 such a decrease cannot be interpreted as complete re-dispersion of the aggregated/agglomerated samples, as here, non-spherical, polydispersed particles were measured. For a reasoning of the decreasing particle size, further analysis would be needed.
Observation of an apparent aggregation upon dispersion in cell culture media was possible for carbon black tattoo ink True Black. The particles are not stable in cell culture medium as the size increases from 276.4 to 964.7 nm. After the 48h incubation, the apparent nanoparticle size strongly decreases to 383.5

nm. This could be an indication that the particles are initially aggregated and then over time stabilised by the proteins in the cell culture medium (Moore et al. 2015).

Unfortunately, no reliable results could be obtained for gold and silver due to their low concentration and hence, low scattering with Brookhaven instrument. Due to the low scattering, measurements with LS instruments were not applicable, hence no signal could be detected. Therefore, further testing with Ultraviolet–visible spectroscopy (UV Vis) was conducted, since the local surface plasmon resonance (LSPR) is characteristic property of plasmonic nanoparticles such as gold or silver (Chanana und Liz-Marzán 2012). DMEM media, however, caused strong interference in the measured spectra. The cell culture media may have absorption bands due to the presence of proteins (280 nm) or phenol red (around 540 nm), which can be seen in the UV/Vis spectra. Here, in this case, the interference is due to the presence of phenol red added to the cell culture media to check the pH. The peak was in the same location as the peak of the particles. Hence, no significant change in LSPR band was observed.

In conclusion, both instruments provided similar results in particle sizes for the tested MNM, and the histograms obtained by the Brookhaven indicated a high sample polydispersity (data not shown), which is in agreement with what is observed with TEM.

Table 7 DLS sizing - stability data summary after 0- and 48-hours following incubation in 1% FBS DMEM + 1% DMSO as used in KeratinoSens™ assay measured with Brookhaven instrument

Sample	Average size (nm)	Uncertainty (nm)	PDI
Nickel(II)oxide (0h)	351.6	3.9	0.214
Nickel(II)oxide (48h)	266.7	1.8	0.166
Titanium(IV)oxide (0h)	313.9	6.8	0.213
Titanium(IV)oxide (48h)	259.7	1.3	0.15
SG-TO50 (TiO_2) (0h)	277.1	5.1	0.13
SG-TO50 (TiO_2) (48h)	272.7	1.6	0.171
Gold (0h)	36.5	0.2	0.284
Gold (48h)	52.0	0.3	0.293
Silver (0h)	41.7	0.7	0.288
Silver (48h)	62.1	0.6	0.232
Carbon black tattoo ink (True Black) (0h)	964.7	18.8	0.156
Carbon black tattoo ink (True Black) (48h)	383.5	3.3	0.267
Carbon black tattoo ink (Pitch Black) (0h)	302.7	2.6	0.117
Carbon black tattoo ink (Pitch Black) (48h)	446.6	5.5	0.304
Pigment red 170 tattoo ink (0h)	246.3	1.5	0.114
Pigment red 170 tattoo ink (48h)	270.8	1.9	0.157

KeratinoSens™

Overall information about the outcome of testing selected MNM in OECD TG 442D (KeratinoSens™ test method) is shown in Table 8.

Table 8 Summary of results testing MNM in KeratinoSens™ assay

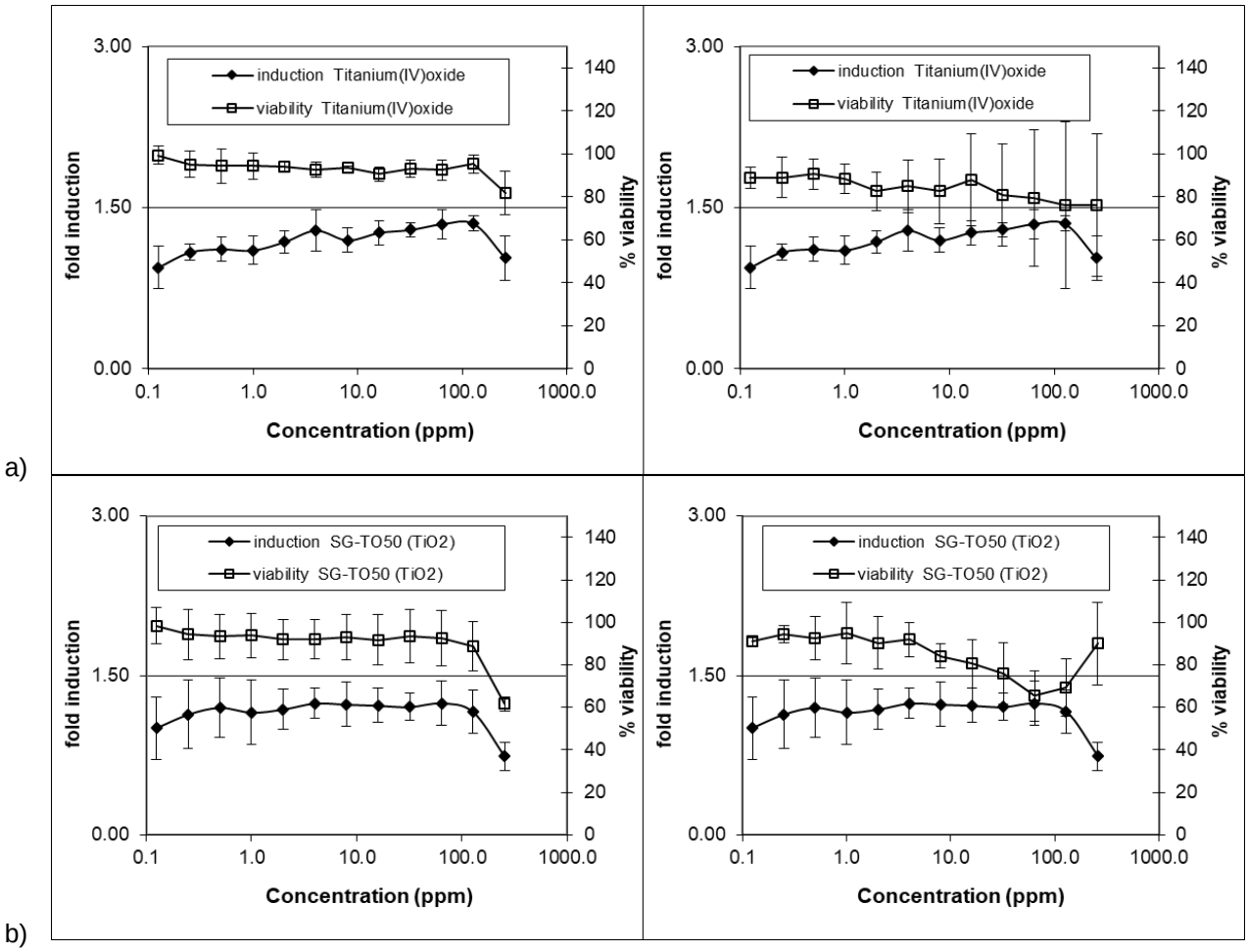
KeratinoSens™	**Reps pos.**	**Overall rating**
Polyethylenglycoldiacrylate nanotubes (PEGDA 575)	0 of 3	NEGATIVE
Nickel(II)oxide	0 of 3	NEGATIVE
Titanium(IV)oxide	0 of 3	NEGATIVE
SG-TO50 (TiO_2)	0 of 3	NEGATIVE
Gold	3 of 3	POSITIVE
Silver	3 of 3	POSITIVE
Carbon black tattoo ink (True Black)	1 of 3	NEGATIVE*
Carbon black tattoo ink (Pitch Black)	1 of 3	NEGATIVE*
Pigment red 170 tattoo ink	3 of 3	POSITIVE
Isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	3 of 3	POSITIVE
Isoeugenol	3 of 3	POSITIVE
Cinnamic Aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	3 of 3	POSITIVE
Cinnamic Aldehyde	3 of 3	POSITIVE
Citral encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	3 of 3	POSITIVE
Citral	3 of 3	POSITIVE

*** In the prediction model of 442D it is specified that if one of 3 repetitions is positive the chemical is rated negative (see Figure 1).**

Testing KeratinoSens™: MNM samples delivered as dry powder

Two qualities of titanium dioxide, SG-TO50 and Titanium(IV)oxide (anatase), were tested. Neither of the two qualities induced luciferase activity over the threshold of 1.5-fold over the entire concentration range (maximal induction over the tested range up to a concentration of 256 µg/mL = I_{Max} = 1.34 and 1.44 respectively, Table 12). For SG-TO50, there was absolutely no dose-response for luciferase induction, while for Titanium(IV)oxide, the luciferase activity increased in a dose-response manner, but remained below the threshold (Figure 3).

Figure 3 Dose response of a) Titanium(IV)oxide and b) SG-TO50 (left: PrestoBlue™ assay; right: MTT assay).



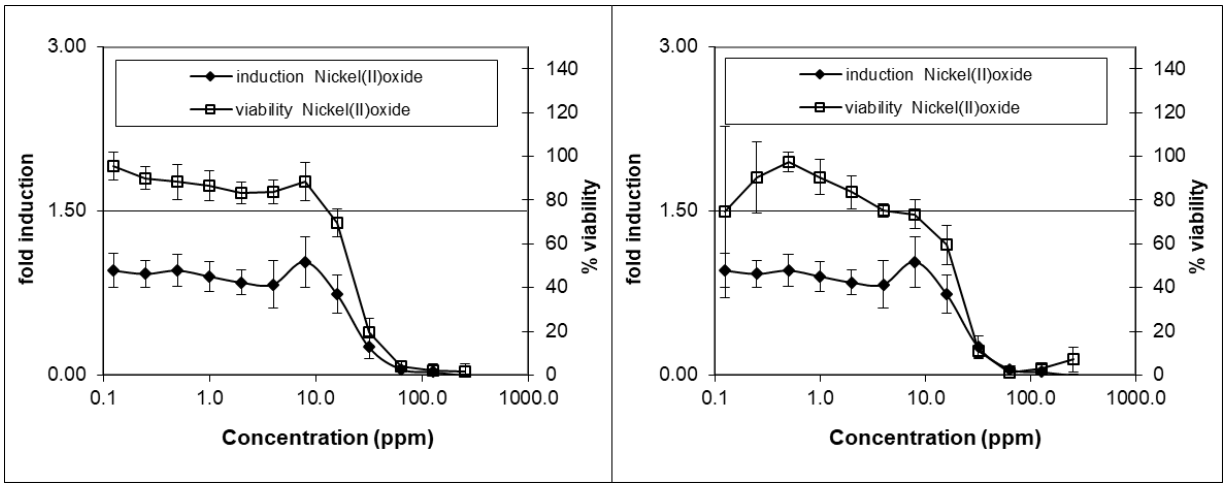
Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL = ppm).

The titanium dioxide particles showed a decrease in cell viability at the top dose of 256 ppm when measured with PrestoBlue™, indicating that significant cellular exposure occurred, however reduction was

less than 50% and hence no IC50 could be determined. The same decrease was also noted for the background luciferase activity, which is typical at cytotoxic doses for chemicals not inducing luciferase, further proving exposure. The results with the MTT assay were subject to more variation and less clear-cut dose-response data.

The nickel(II)oxide particles did not induce the luciferase gene above the threshold (I_{Max} = 1.08). These particles were significantly more cytotoxic with an IC50 of 18.6 µg/mL (Figure 4).

Figure 4 Dose response of nickel(II)oxide (left: PrestoBlue™ assay; right: MTT assay).



Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL = ppm).

Thus, all three nanoparticle preparations delivered as powder and dispersed according to the NANOGENOTOX dispersion protocol for NANoREG did not induce the luciferase gene above the threshold of 1.5 in all three repetitions and are thus rated negative in the KeratinoSens™ assay in all three independent repetitions conducted, and hence also no EC1.5 values could be derived.

Testing in KeratinoSens™ – MNM samples delivered as dispersion

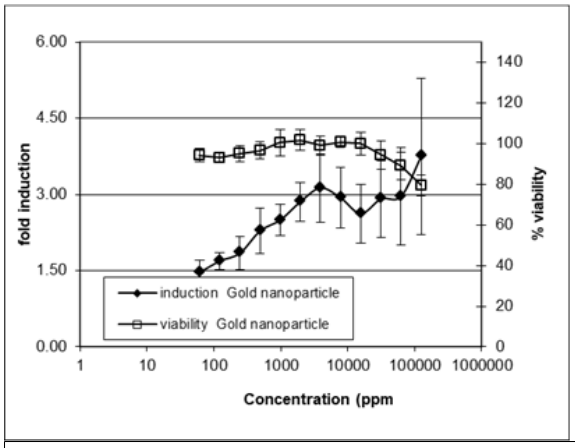
Silver and gold were delivered as diluted dispersion stabilised with sodium citrate and citrate buffer, respectively. While for silver a concentration in weight is given (0.02 mg/mL), for the gold dispersion only the optical density and approximate number of particles are given. Therefore, these two preparations were directly diluted and tested. The data in the tables and figures are all given in relation to µg/mL of the original dispersion (nominal), and not to µg/mL of actual particles (analytical).

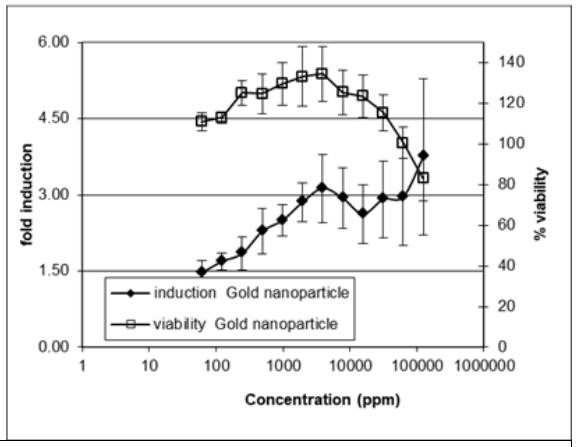
For the silver the maximal concentration of the dispersion tested is 250,000 µg/mL equalling to 25%. As for the silver dispersion a concentration is given (0.02 mg/mL), we can calculate the maximal test concentration for the actual particles in a weight/mL basis and this would be 5 µg/mL, hence significantly lower as compared to the particles delivered as powder and below the typical maximum test concentration required by the prediction model in the SOP of KeratinoSens™ (200 µg/ml) to rate chemicals as potential negatives, but since the result was positive and since cytotoxicity indicated sufficient exposure, this limited maximal concentration does not affect the result of KeratinoSens™.

The full dose response curve is shown in Figure 5 for gold and silver, respectively. Both nanomaterials showed a clear and dose-dependent decrease in cellular viability at the top concentrations tested, although viability dropped not below 50% and hence no IC50 was calculated. Still this drop in viability shows that the nanomaterials were concentrated enough to reach maximal exposure and partial toxicity.

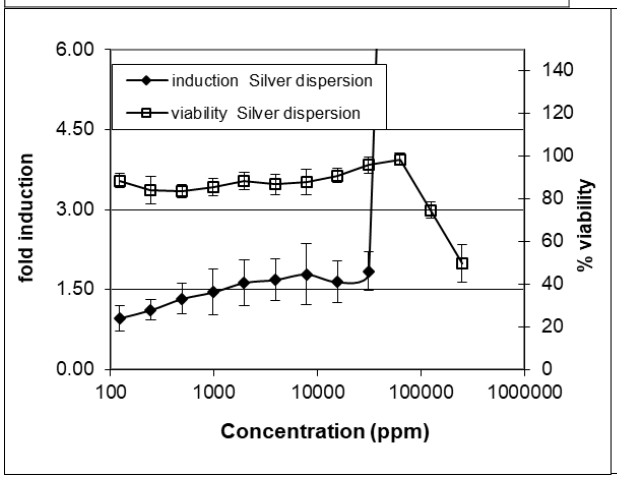
Figure 5 Dose response of a) gold and b) silver (left: PrestoBlue™ assay; right: MTT assay).

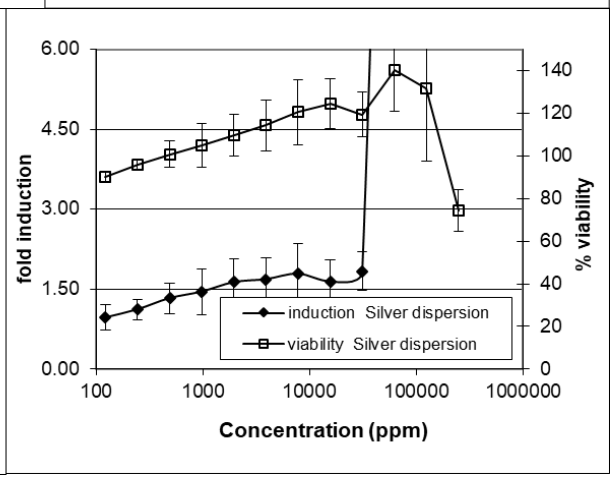
a)





b)





Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (equivalent to µg/mL) related to the purchased dispersion, thus 10,000 ppm refers to 1% of the original dispersion.

Note: As these materials were purchased as diluted dispersions, the maximal concentration of the solid matter thus may be lower as the 200 ppm required by the prediction model. But as the materials were positive clearly below the maximal test concentration, testing below the maximal concentration of the SOP is sufficient, also because cytotoxicity indicates higher concentration would lead to more toxic effects.

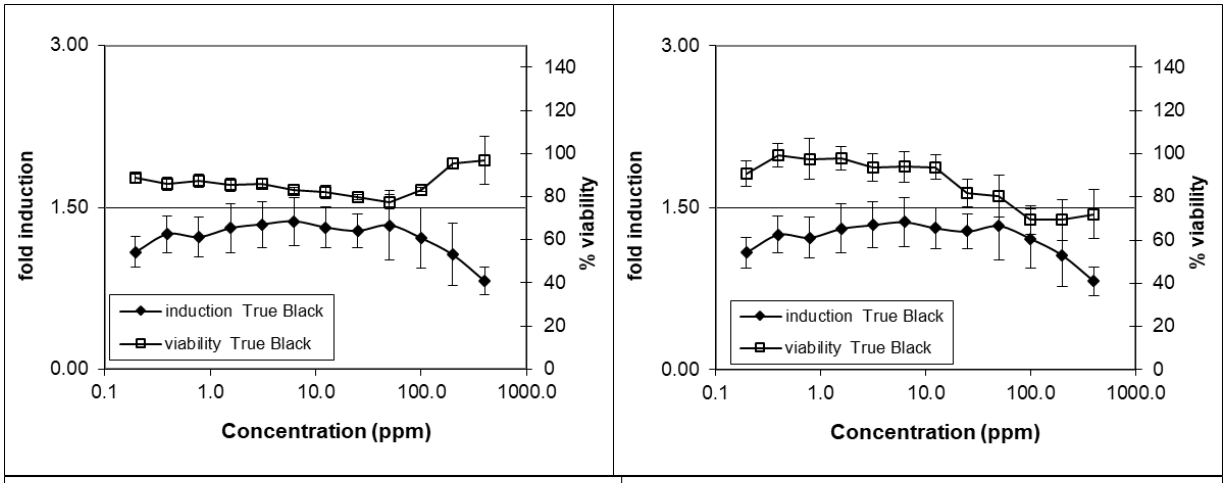
Testing in KeratinoSens™ – Tattoo inks

Three Tattoo inks were tested in the KeratinoSens™ assay, as commercially purchased. The two carbon black tattoo inks (True Black and Pitch Black) were received as dispersions, while the pigment red 170 tattoo ink was received as a powder. The carbon black tattoo inks were tested up to 400 µg/mL on a weight per volume basis calculated based on the full ink dispersion as received. This approach was chosen, since no indication is available on the amount of solid material in the ink. Pigment red 170 tattoo ink was dispersed according to the NANOGENOTOX dispersion protocol for NANoREG (NANoREG) and tested up to 256 µg/mL.

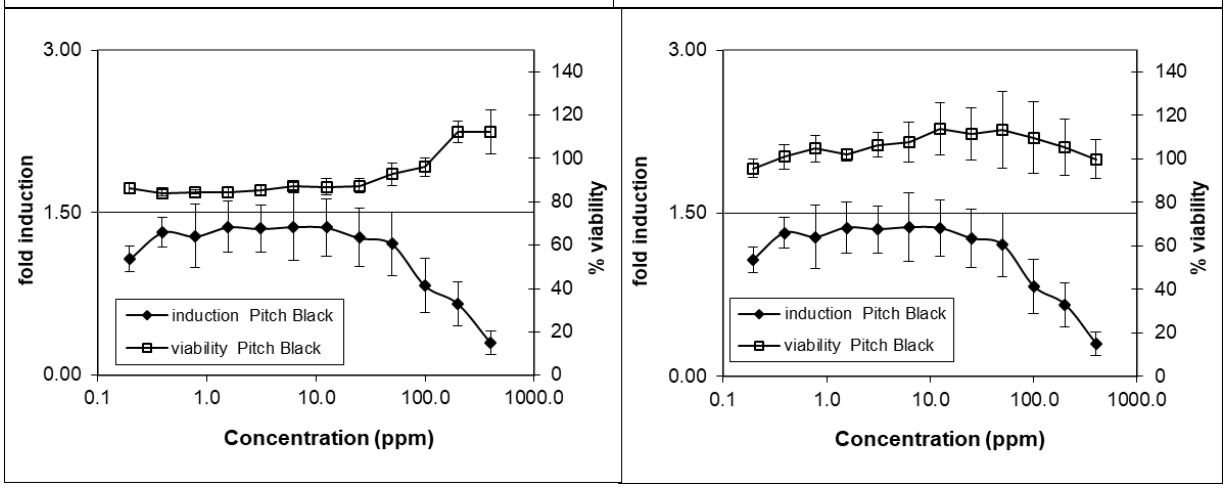
The full dose response curve is shown in Figure 6 for tattoo inks carbon black True Black, Pitch Black, and pigment red 170 tattoo ink respectively. No significant cytotoxicity was shown for all three inks at the tested concentration range, both when assessed with the PrestoBlue™ and with the MTT assay. For pigment red 170 tattoo ink, a strong interference with the MTT assay was observed, the MTT signal was suppressed over the entire concentration range. However, no interference was observed in the parallel assay with PrestoBlue™.

Figure 6 Dose response of tattoo inks a) carbon black True Black, b) carbon black Pitch Black, and c) pigment red 170 (left: PrestoBlue™ assay; right: MTT assay).

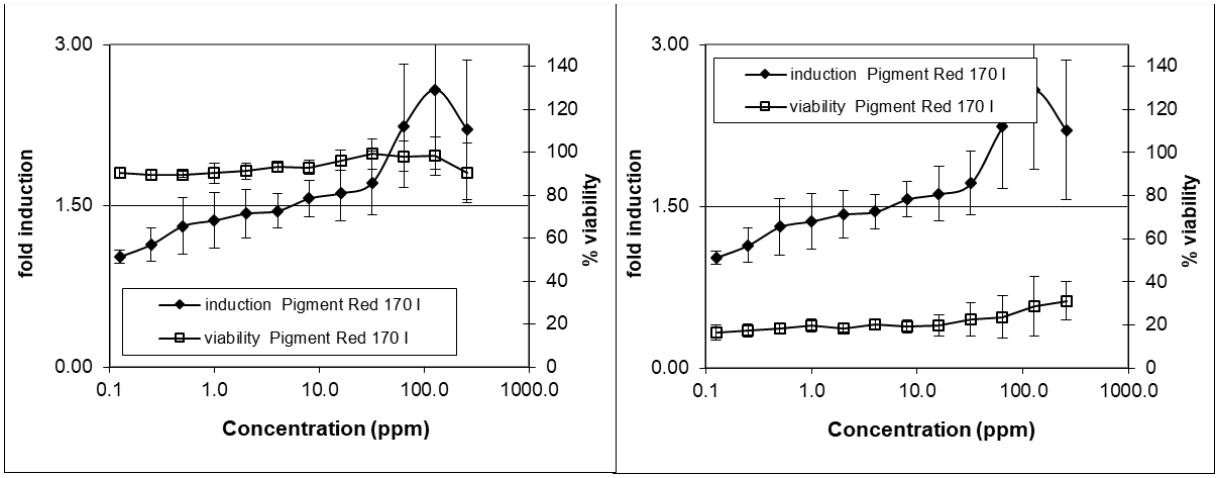
a)



b)



c)

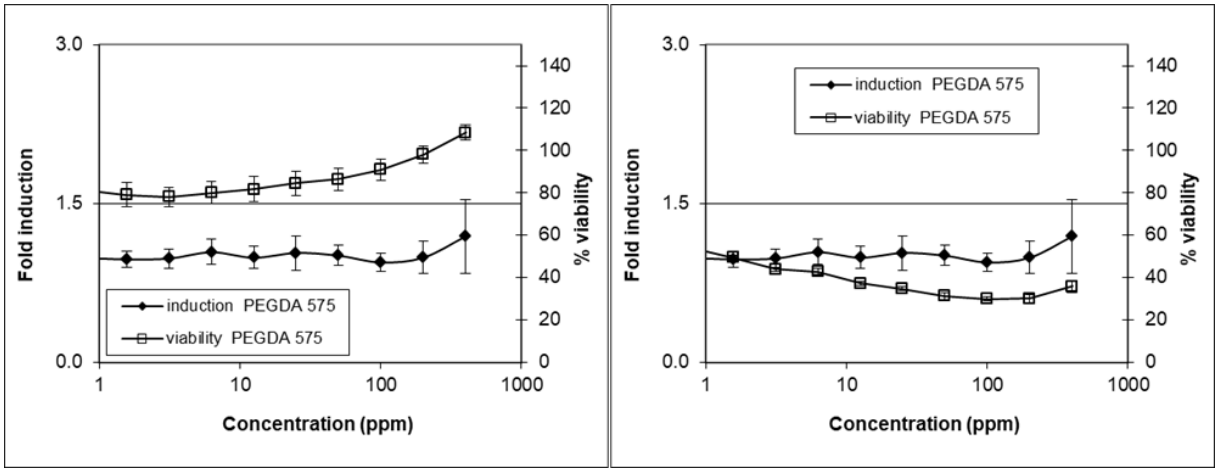


Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL = ppm) of the complete ink dispersion as obtained.

Testing in KeratinoSens™ – Testing of Polyethylenglycoldiacrylate nanotubes

As until now most nanomaterials tested in KeratinoSens™ were of inorganic nature, we were also interested in more potential materials made from organic polymers. One example are Polyethylenglycoldiacrylate nanotubes as described by Newland et al. (2018). The material polyethylenglycoldiacrylate nanotubes (PEGDA 575; Mw around 575 Da, 10 PEG units) was obtained from the authors. It was dispersed in test medium by sonication in a sonication bath as recommended by the authors and dissolved at final conc. of 1.6 mg/mL in 4% DMSO. Dilutions of this stock dispersion (50 µL) were then added to cells with 150 µL test medium (final maximal conc. 400 ppm and 1% DMSO). As shown in Figure 7, this material was negative in KeratinoSens™ up to the top dose. Thus, while acrylates prior to polymerisation are strong sensitisers, the fully polymerised nanotubes are negative in KeratinoSens™. The MTT data indicate some assay interference: While the cellular viability appeared not to be affected when assessed by PrestoBlue™ assay, there was a strong suppression of the MTT signal, despite the fact that the stable luciferase signal also did not indicate loss of cellular viability. While it cannot mechanistically be explained why the PEGDA tubes interfered with the MTT assay, this result confirms that adding the second viability assay with PrestoBlue™ performed in solution is a valid addition to reduce assay interference with the MTT cytotoxicity assay as also observed for pigment red 170 tattoo ink.

Figure 7 Dose response curve of polyethylenglycoldiacrylate nanotubes (PEGDA 575) (left: PrestoBlue™ assay; right: MTT assay). The right side shows the strong suppression of the MTT signal.



Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL).

Testing in KeratinoSens™ – Positive controls, chemicals encapsulated in polycaprolactone particles

As a positive control, three well-known natural skin sensitisers occurring in essential oils and fragrances (citral, isoeugenol and cinnamic aldehyde) were encapsulated in poly-Ɛ-caprolactone particles. The particles were produced by EZUS-Lyon (France) according to Cortial et al. (2015) and delivered as dispersions, and they were tested in parallel in comparison with equal concentrations of the free sensitisers.

All three polycaprolactone particle preparations as well as the free test molecules induced the luciferase gene above the threshold of 1.5 in all three repetitions and are thus rated positive in the KeratinoSens™ assay. Furthermore, the EC1.5 values are very similar for the free and the encapsulated material, as shown in Table 9. This indicates that the exposure from the particles and from the free material is very similar. Whether this is due to passive leaking of the samples already during storage and dilution or whether it is due to efficient transfer of the positive control sensitisers from the particles to the cells during the test is not entirely clear.

Table 9 Chemicals encapsulated in poly-Ɛ-caprolactone particles - Luciferase determinations in comparison to the free molecules.

Test substance	Rep 1 (µg/mL)	Rep 2 (µg/mL)	Rep 3 (µg/mL)	Geometric Mean (µg/mL)
Citral encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	2.96	2.17	2.14	2.40
Citral	0.94	1.62	3.22	1.70
Cinnamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	1.12	2.39	2.26	1.82

Cinnamic aldehyde	1.43	2.26	2.30	1.95
Isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	1.04	1.80	1.06	1.25
Isoeugenol	0.95	1.94	2.09	1.57

EC1.5 value is shown as the concentration in µg/mL inducing the luciferase activity 1.5 fold up to a concentration of 200 µg/mL.

Testing in KeratinoSens™ – short time exposure protocol

In the KeratinoSens™ protocol, the cells are exposed to the test items for a prolonged time (48 hours). It was initially hypothesised, that contact to particles could become increasingly toxic to the cells due to particle deposition on the adherent cells over time leading to high local concentration. Thus, a potential modification of the protocol is to expose the cells for 4 hours only and then wash away the particles and to continue incubation up to the full incubation time of 48 hours. In this post-exposure incubation time, gene expression due to absorbed or adhering particles or due to the already induced Nrf2 pathway could still occur.

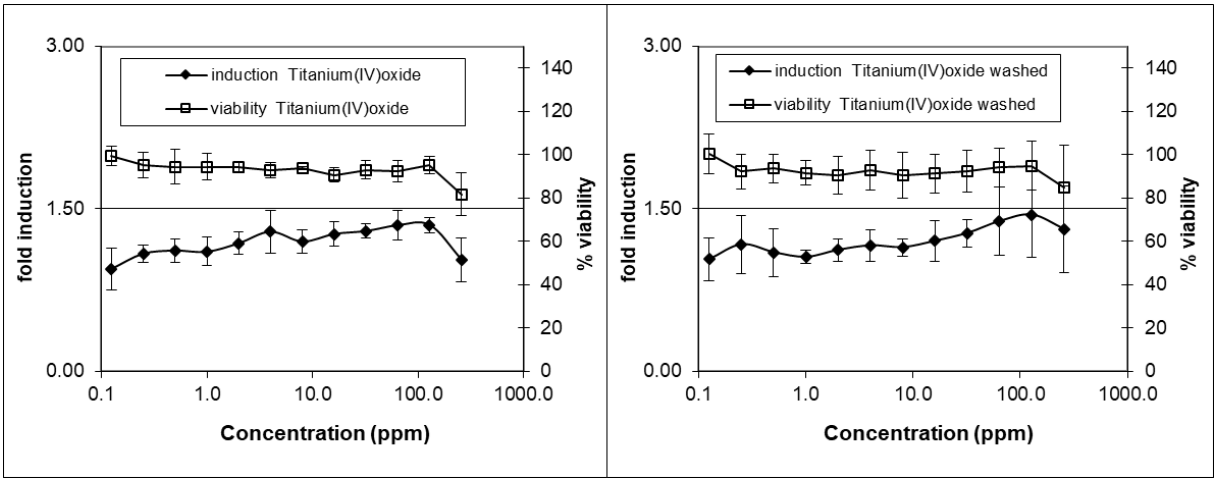
Below data are shown for this refined protocol in Figure 8 for both titanium dioxides and nickel(II)oxide. Both qualities of titanium dioxide particles showed very similar result as with the standard protocol. No induction of luciferase above the threshold and weak cytotoxicity with concomitant reduction of background luciferase at the top concentration. These results indicate that despite the difference in exposure time, the cytotoxicity profile was found to be similar.

Similarly for the nickel(II)oxide, the washed-protocol showed a similar result as observed in the standard protocol, with cytotoxicity and an IC50 of around 30 µg/mL. This indicates that despite the difference in exposure time, the cytotoxicity profile is similar.

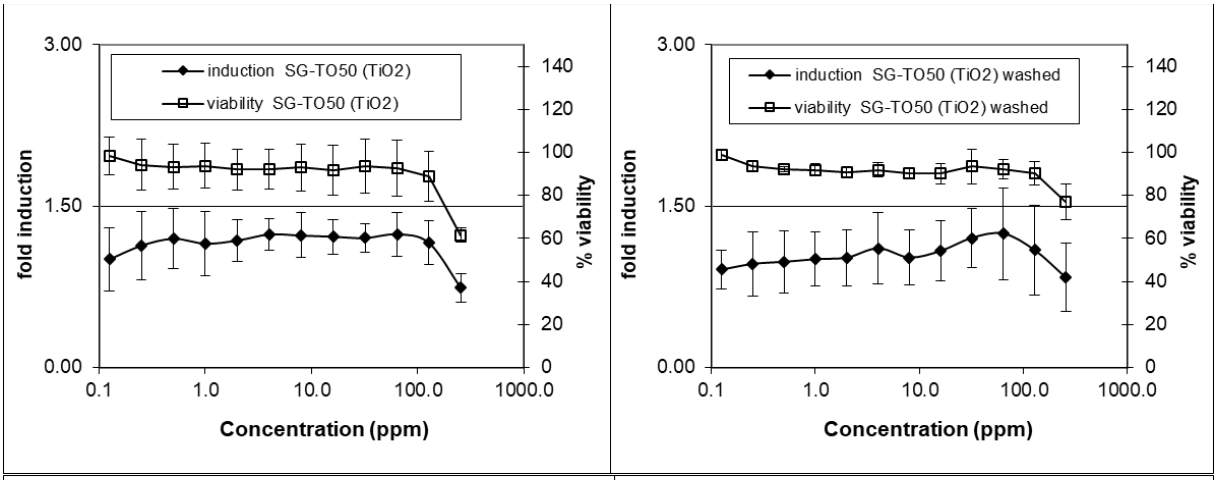
Figure 8 Short time exposure (4 hours) in KeratinoSens™ of a) titanium(IV)dioxide, b) SG-TO50 titanium dioxide, and c) nickel(II)oxide. Viability was assessed with the PrestoBlue™ assay.

A comparison is shown for the standard exposure time (left) and 4 h exposure time followed by washing and post-incubation (right)

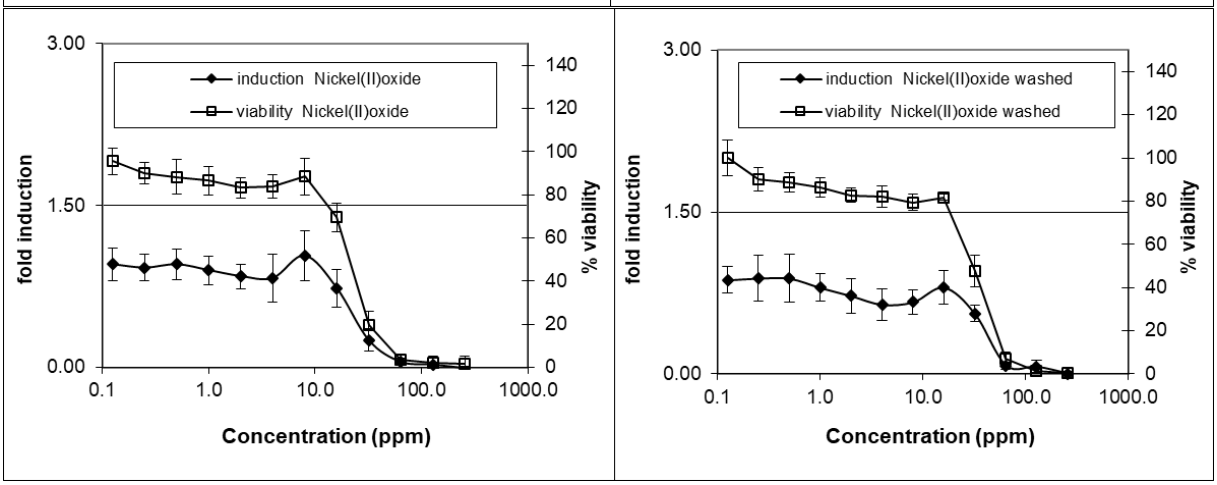
a)



b)



c)



Cells were treated with the particles for 4 hours and then the medium with the particles was removed and replaced with fresh medium containing 1% DMSO and incubation was continued for 44h. Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL).

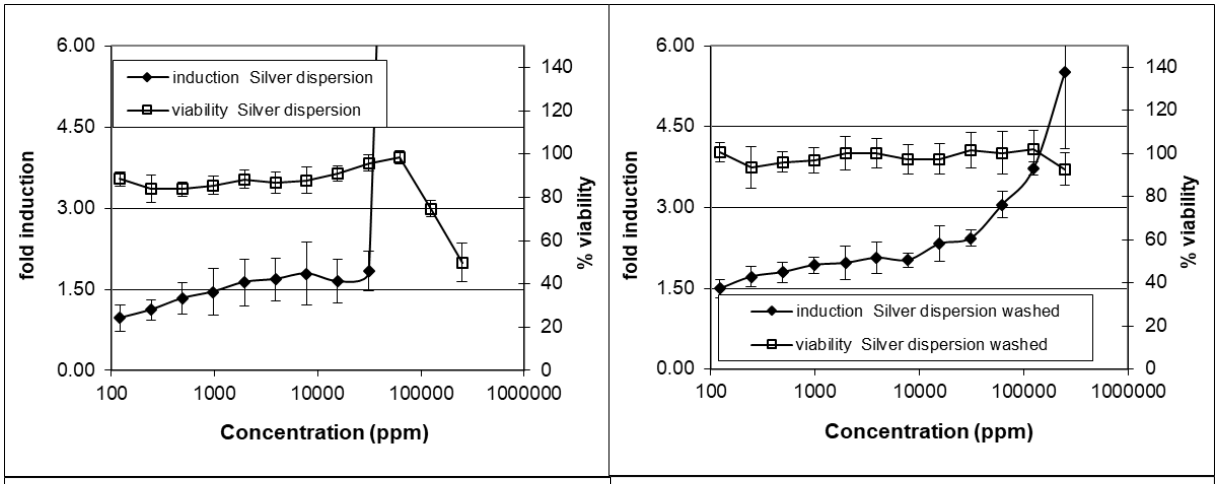
Both silver and gold were again clearly positive in all three repetitions with exposure for only 4 hours followed by a post-incubation period (Figure 9). For silver, the threshold was even crossed at clearly lower concentration, although it is not clear whether this is due to the fact that the whole dose-response curve was shifted to slightly higher induction. What is clear is that for silver the cytotoxicity was strongly reduced by the short incubation protocol.

For gold, the curves for both protocols are very similar, indicating that exposure is quite similar for both application protocols, and that sufficient exposure occurs with a shorter contact time.

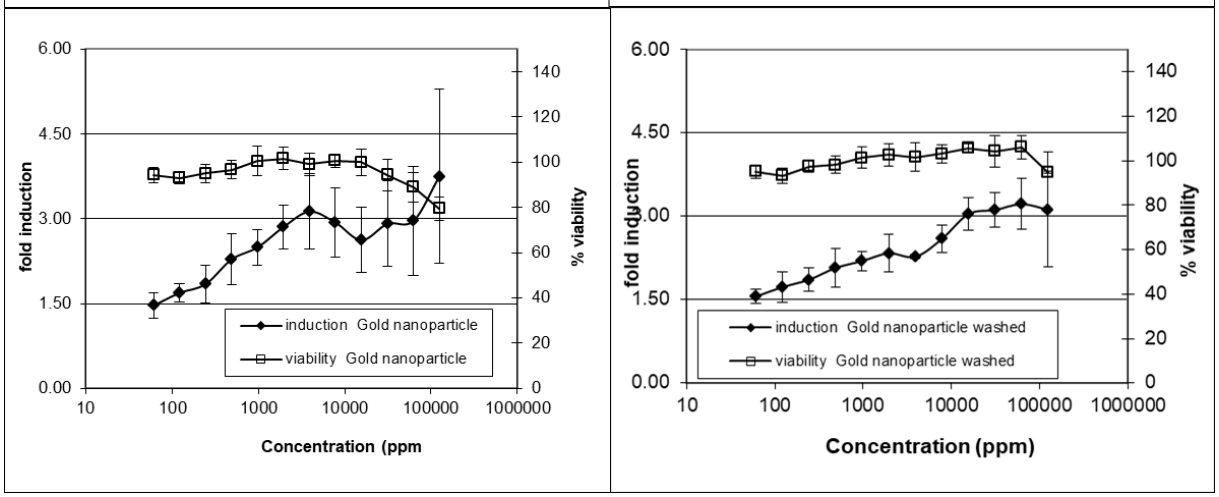
Figure 9 Short time exposure (4 hours) of MNM in KeratinoSens™ a) silver and b) gold. Viability was assessed with the PrestoBlue™ assay.

A comparison is shown for the standard exposure time (left) and 4h exposure time followed by washing and post-incubation (right)

a)



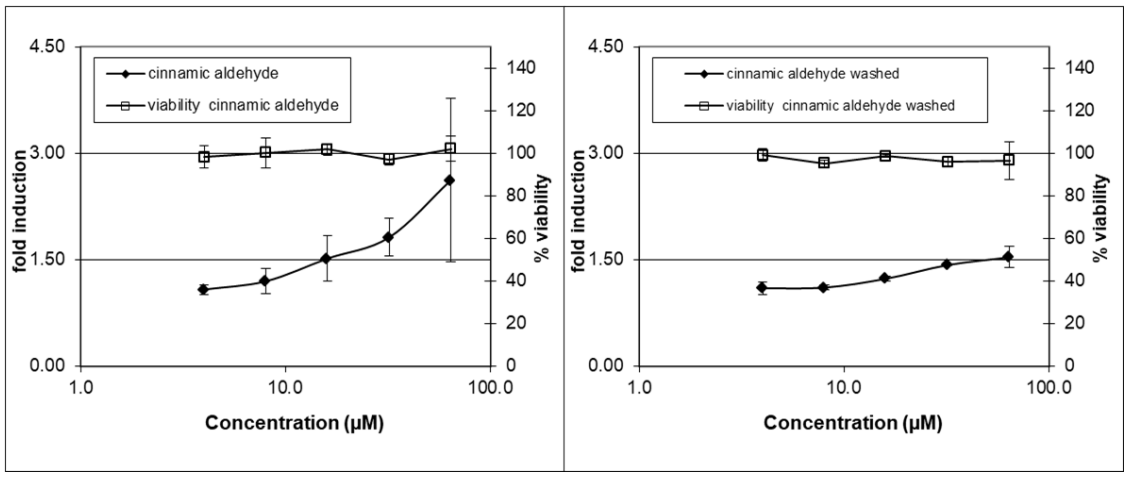
b)



Cells were treated with the particles for 4 hours and then the medium with the particles was removed and replaced with fresh medium containing 1% DMSO and incubation was continued for 44 hours. Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL). For reference, the data with the standard assay (from Figure 5) are given on the left side, while the data with shorter exposure time (the washed protocol) is shown on the right side of the panel.

For the positive control cinnamic aldehyde, a much weaker activity was observed with 4 hours contact time as compared to the full contact time (Figure 10). This is in line with experiments conducted during KeratinoSens™ SOP definition, when it was found that 4 hours contact time is not sufficient for full expression of the response when testing typical low molecular weight skin sensitisers, even if normally Nrf2 activation is reported to occur within hours.

Figure 10 Short time exposure in KeratinoSens™ with positive control cinnamic aldehyde.



For reference, the data with the standard assay are given on the left side, while the data with shorter exposure time (the washed protocol) is shown on the right side of the panel. Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in ppm on a weight per volume basis (µg/mL).

Testing in KeratinoSens™ – Testing of leachates from nanomaterials

In another iteration of the testing, the response of the KeratinoSens™ assay to leachates from nanomaterials was tested. This follows the principle in general used for e.g., medical device toxicity and compatibility testing, where particles or devices are incubated with e.g., test medium, and effects of any leaching substance is assessed by testing the leachate. The principle behind this was to identify a potential solution for non-dispersible MNM. Thus for the inorganic particles titanium dioxide SG-TO50 and the nickel(II)oxide leachates were prepared according to "ISO 10993-12:2012; Biological evaluation of medical devices – Part 12: Sample preparation and reference materials".

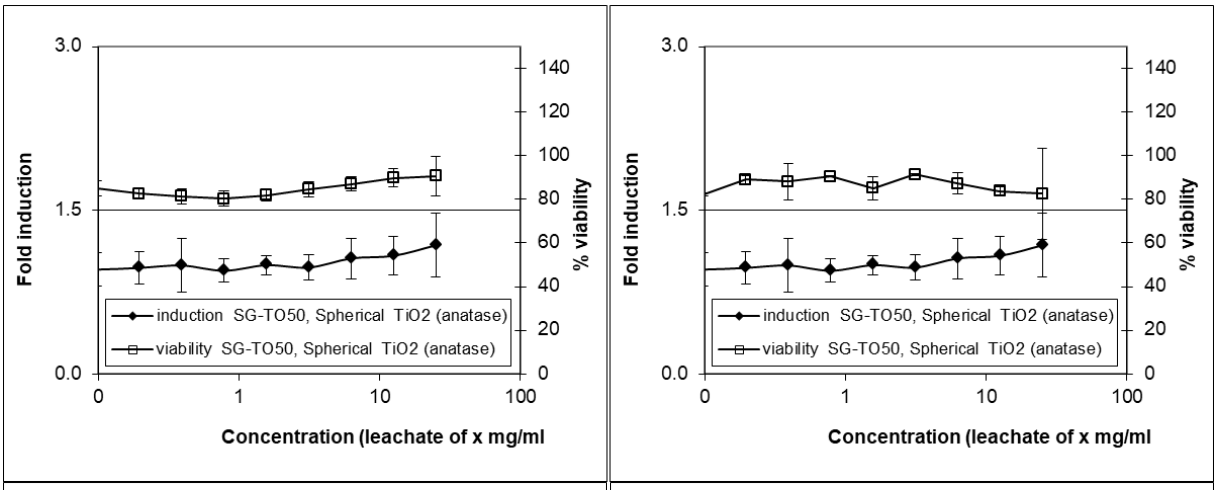
Thus, 1 g of the nanomaterials were added to 10 mL cell culture medium (with Penicillin Streptomycin and 4% DMSO) and shaken at 250 rpm for 72h at 37°C. The dispersions were centrifuged at 5000 rpm for 10 min and the supernatant was directly used for dilution series. Dilutions of this stock solution (50 µL) were then added to cells with 150 µL test medium. Thus, the top concentration corresponds to a leachate from 25 mg material per mL.

As shown in Figure 11, also leachates of the titanium dioxide SG-TO50 sample were clearly negative in KeratinoSens™, similar to the situation when the particles are tested directly.

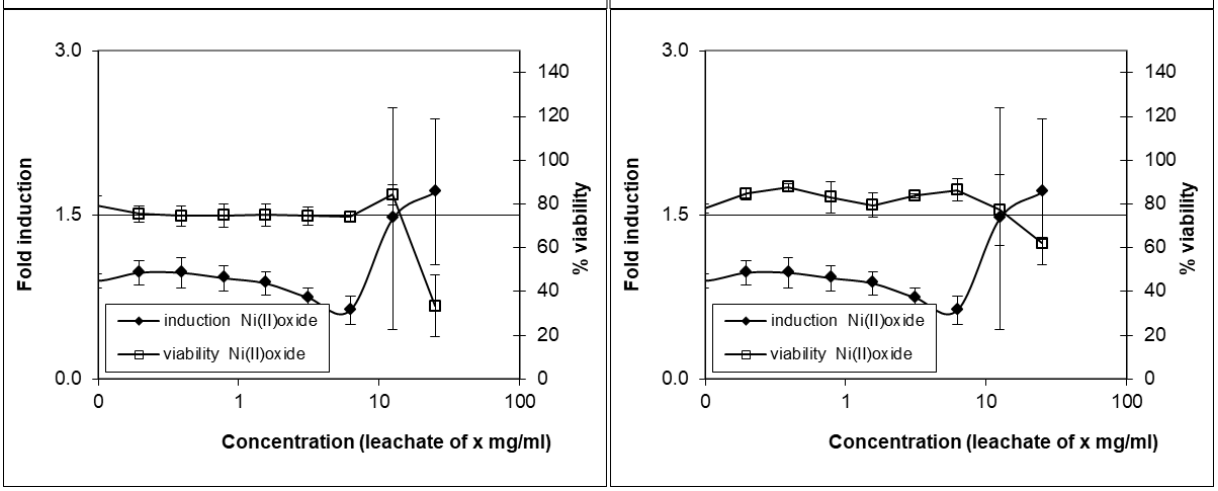
For the leachates from nickel(II)oxide, at the top concentration induction of luciferase concomitant with cytotoxicity were observed (Figure 11). At the second highest concentration, a high variability was observed, in one repetition, the luciferase signal was positive but it was negative in the two other repetitions. Since there is no reproducible luciferase induction at non-cytotoxic concentration, thus also this leachate is rated negative, although the dose-response and the variability indicate that it is close to the decision threshold.

Figure 11 Leachates of MNM tested in KeratinoSens™ of a) SG-TO50 titanium dioxide and b) nickel(II)oxide (left: PrestoBlue™ assay; right: MTT assay).

a)



b)



Leachate from MNM after 72 hours shaking in cell culture medium tested in KeratinoSens™. Shown are averages and standard deviations of three independent experiments each conducted in triplicate at each concentration. Concentrations are given in mg/mL, whereby the value indicates from how many mg of particles per mL the leachate is derived.

Testing in KeratinoSens™ – Acceptance criteria and positive control

Cinnamic aldehyde was run in all experiments and in all three repetitions of each experimental series. The detailed results for the positive control are reported in Table 15 in the Annexe of this study report.
The final results as reported here were generated in three independent experimental series, hence a total of nine runs were performed.

Comparison of *in vitro* results to existing *in vivo* information from the literature

Table 10 gives an overview of the comparison of the results from tested MNM in KeratinoSens™ within this study and the available information on the corresponding MNM in literature mainly from *in vivo* testing. Except for gold, which was selected based on scientific evidence that keratinocytes exposed *in vitro* to gold MNM (5 nm) activate the Nrf2 pathway (Goldstein et al. 2016).

It should be noted, that the literature evidence that TiO_2 MNM are sensitisers is rather weak (Auttachoat et al. 2014). Thus, TiO_2 MNM tested under the standard Local Lymph Node Assay (LLNA) conditions was negative, while irritant effects were noted. Only by sub-cutaneous injection of the TiO_2 MNM, an induction of cell proliferation just above the threshold of 3 was achieved and the authors concluded that these results suggest *"irritant and/or potential hyper-sensitivity responses following dermal administration of nano-TiO_2 to compromised skin"*. In the guinea pig test cited in the OECD Titanium Dioxide dossier (OECD Environment Directorate 2016f), only 1/17 animals (6%) showed a reaction and this was only a grade 1 erythema. In the report this result is cited with the conclusion *"weak sensisitizer"*. However, as the GHS criteria are not met, we consider the preparation to be also negative in the Buehler assay. In this regard the obtained KeratinoSens™ results can be considered as correct-negatives.

Nickel in the form of an easily soluble salt as used in patch testing or as metallic nickel with prolonged skin contact leading to leaching of metal ions is an important human sensitiser, but the true *in vivo* result for the poorly soluble nickel(II)oxide is not known. Hence the negative *in vitro* result obtained is a false-negative to nickel in general – but no firm conclusion for nickel(II)oxide can be made.

Further, both carbon black tattoo inks were selected to be tested within this study as negative control for tattoo inks, although Høgsberg et al. (2011) has shown that this tattoo ink could be a weak skin sensitiser. However, this could be the case if the carbon black includes organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) (Bil et al. 2018). Nevertheless, carbon black tattoo inks were included based on a weight-of-evidence approach in the *in vitro* to *in vivo* comparison as negative *in vivo* tattoo ink (Bernatikova et al. 2018, Scientific Committee on Consumer Safety (SCCS) 2013, Laux et al. 2016, Serup et al. 2015). The negative results for both black tattoo ink preparations are thus well aligned with the clinical observations, that allergic reactions to black tattoo inks normally do not occur unless a PAH contaminated ink is used.

For pigment red tattoo ink 170, the most frequent allergic reactions are reported (Serup et al. 2015), although the true sensitising substance/pigment in most cases is not known. However, the here tested pigment red 170 tattoo ink was repeatedly found by analytical means in allergic lesions of allergic individuals, giving supporting evidence that it could be the sensitising species (Serup et al. 2020).

Since KeratinoSens™ is based on keratinocytes activation, due to Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes, we reasoned that gold MNM could represent a good positive control, even if evidence was based on cellular pathway activation rather than on *in vivo* experiments. Yet the data indicate that indeed the gold particles are strongly positive in the KeratinoSens™ assay.

An *in vitro* to *in vivo* comparison was not performed for PEGDA 575 because, even if generally considered biocompatible (Newland et al. 2018), no data regarding its sensitisation potential was found in the literature.

Table 10 MNM comparison on *in vitro* results from KeratinoSens™ testing and information from literature.

NM name	Data source (literature)	Result Literature	Result TG 442D	Comparison (test results/literature information)
Titanium(IV)oxide	LLNA	Stimulation index (SI) ≥ 3; but only with intradermal injection; negative in standard LLNA (Auttachoat et al. 2014)	Negative	Ambiguous[1)]
SG-TO50 (TiO2)	OECD TG 406	Erythema (weak/ambiguous sensitizer); (Grade 1 erythema in 1/17 animal); negative by GHS criteria (OECD Environment Directorate 2016f)	Negative	Ambiguous[1)]
Nickel(II)oxide	Occupational exposure to metallic nickel	Allergic reaction to metallic nickel and soluble nickel salts; no data for nickel(II)oxide (Journeay und Goldman 2014)	Negative	Ambiguous[2)]
Gold	*In vitro* exposure to keratinocytes and macrophages	Induction of Nrf2 (Goldstein et al. 2016)	Positive	Yes (*in vitro – in vitro*)
Silver	OECD TG 406	Erythema (weak sensitizer) in one guinea pig (1/20); negative by GHS criteria (Kim et al. 2013)	Positive	Ambigous
Carbon black (True black)	Epidemiological studies	Negative control (Bernatikova et al. 2018, Bil et al. 2018, Serup et al. 2015)	Negative	Yes[3)]
Carbon black (Pitch Black)	Epidemiological studies	Negative control (Bernatikova et al. 2018, Bil et al. 2018, Serup et al. 2015)	Negative	Yes[3)]
Pigment red 170 tattoo ink	*In vivo* analytical studies in allergic individuals	Allergic reaction (Serup et al. 2015, Serup et al. 2020)	Positive	(Yes)[4)]

[1)] weak *in vivo* sensitisation was concluded in corresponding reports, but *in vivo* data are negative under standardized conditions (topical LLNA) and the GHS decision criteria (Buehler assay)

[2)] Compared to positive clinical tests with soluble nickel salts and long-time exposure to nickel leaching alloys and solid metal, the negative result for nickel(II)oxide is considered a false-negative. However, as nickel(II)oxide may have a different solubilisation behaviour this *in vivo* to *in vitro* comparison cannot directly be made.

[3)] Black tattoo ink is mainly composed of pigments of natural origin, such as carbon black (Pigment Black 6/7). This pigment consists mainly of elemental carbon (>97%). The Scientific Committee for Consumer Safety (formerly the SCCP) concluded, in their scientific opinion, that this carbon black pigment, when considered in its nanostructured form, can be regarded as being safe at concentrations up to 10% in consumer products (Bil et al. 2018), From the clinic, normally

allergies are only reported to red (and green and blue) tattoo inks, but not black tattoo inks (Serup et al. 2015).

4) Allergic reactions to red tattoo ink are often seen (Serup et al. 2015) and analytical investigations found pigment red 170 tattoo ink as a pigment in many lesions (Serup et al. 2020) – however this is only an indirect association, as there is no direct *in vivo* sensitisation assay with the pure pigment available.

8 Study summary and conclusions

Main questions and their answers in a nutshell:

- **Is the TG technically applicable?**
  Yes, OECD TG 442D (KeratinoSens™ test method) is from a technical point of view applicable for the testing of MNM. However, it has to be stated that it was not possible to make any further assumption about the relevance for the *in vivo* comparison, due to the scarce availability of data for MNM that has been tested *in vivo*, especially the little data with standard OECD tests. Nevertheless, the work conducted within this project can be seen as a starting point for further work in regard of MNM safety testing for skin sensitisation.
- **Which nanomaterials are suitable for testing?**
  Different MNM were selected to be tested within this project. The selection of the MNM has been based on an extensive literature review and was dependant on information about the skin sensitising potential of these MNM. In total 12 inorganic and organic MNM were selected (9 test materials; 3 controls). Testing with KeratinoSens™ was from a technical point of view possible with all of the selected MNM. Sample preparation proved to be a critical step when testing MNM. In this study already existing SOPs from the previous EU project NANoREG[3] were used for sample preparation for particles which were in powder form.
- **Are there nanomaterials that were not possible to test?**
  During the practical part of this study there were no MNM identified that could not be tested. However, it has to be noted that the sample number of twelve MNM is relatively small in comparison of the variety of MNM. Therefore, we cannot conclude on whether there is one MNM group that cannot be applied to KeratinoSens™. However, in the meantime further groups of MNM have been successfully tested as published in recent studies (Kim et al. 2021a, Kim et al. 2021b, Kim et al. 2020b, Kim et al. 2021c, Lee et al. 2021) and as part of ongoing work at University of Trieste, which is not yet published (contacts are Dr. Marco Pelin, Prof. Aurelia Tubaro, and Prof. Maurizio Prato).
  Overall, the critical step might be the sample preparation. If a MNM cannot be dispersed to be tested in the respective media for KeratinoSens™ it cannot be tested in this assay. But this fact would not be something MNM specific, as this is also the case for "ordinary" chemicals. However, we have done leaching experiments (according to "ISO 10993-12:2012; Biological evaluation of medical devices") with some of the selected MNM (see under Testing in KeratinoSens™ – Testing of leachates from nanomaterials) and this showed that testing leachates could be another option to gain evidence for sensitisation risk from MNM constituents.
- **What has to be adapted to use the TG for nanomaterials?**
  According to the experience gained during the testing of selected MNM with the

[3] https://www.rivm.nl/en/about-rivm/mission-and-strategy/international-affairs/international-projects/nanoreg/work-package

KeratinoSens™ and based on the discussion within the two expert workshops in December 2019 and 2021 some recommendations can be made in regard to the dispersion protocols, needs for endotoxin assays, potential to include leaching experiments, and the role of DMSO as a mediator to assist nanomaterial penetration into cells. Further, the potential for nanomaterials to interact with detection methodologies also brought in the possibility to use two different colorimetric cytotoxicity assays.

- **Are protocol changes needed to test nanomaterials?**
  Some of the recommendations made under the previous point, can be directly addressed by adaptation of the SOP of the KeratinoSens™ test method, e.g., viability assessment.

The main question addressed with the experimental work carried out here and discussed within this report is the technical applicability of the KeratinoSens™ test protocol for testing the selected MNM described in this study report.

The plausibility of results was only assessed in a descriptive way of comparing the *in vivo* information from literature and the outcome obtained by testing in KeratinoSens™ of the selected MNM in this study and shown in Table 10. However, this basic comparison revealed that in future more information will be needed for a more complex comparison for *in vitro* to *in vivo* correlation and the available *in vivo* data is very scarce.

In the standard protocol of KeratinoSens™, low molecular weight chemicals are tested. They are routinely dissolved in DMSO and then added to the cell culture medium. Alternatively, the SOP also allows directly dissolving the chemicals in cell culture medium or another appropriate solvent.

Here the NANOGENOTOX dispersion protocol from NANoREG was implemented (NANoREG). This protocol can easily be combined with the KeratinoSens™ protocol, and the dispersions obtained are diluted to prepare a master plate with 12 concentrations following the standard KeratinoSens™ protocol. However, the characterisation exercise showed that the dispersions of the different nanomaterials were not stable as they agglomerated/aggregated over time. Discussions on what the cells were exposed to (e.g., actual dose and form – particle or free molecules) will arise, especially in the regulatory context. Therefore, standardised test guidelines and guidance documents will help. Such are currently under development in the OECD Test guidelines Programme (new TG's in section 1 will have to be cited once published by the OECD) and in various EU Projects, to reduce some uncertainties/questions about the outcome of such *in vitro* tests.

In general, the response of luciferase is more pronounced (higher dynamic range) in the presence of 1% DMSO, the KeratinoSens™ protocol requires that the final concentration of DMSO in the test well is always at 1%, regardless of the initial solvent used to dissolve the test items. This approach was therefore also followed here. No DMSO was added to prepare the initial dispersions or dilutions, but upon substance and medium addition, this final level of DMSO was adjusted. With this approach, no deviation from the protocol is made, and the dynamic range of the test and especially the performance of the positive control required for test acceptance are not changed. Currently it is not known whether the low DMSO concentration does affect the uptake of particles into the cells.

For those chemicals delivered as solid, a maximal test concentration of 256 ppm was applied. The prediction model uses 200 ppm as a cut-off: Chemicals which do not induce the luciferase up to 1.5-fold up to 200 ppm are rated negative. This cut-off was defined in the original publication (Emter et al. 2010) based on testing of the so called Silver list. Thus, the required maximum test dose was achieved for all powders.

For all the test items delivered already as a dispersion, the test is straightforward and the protocol could be followed by simply diluting the dispersions in cell culture medium and adding them together with cell culture medium containing DMSO to achieve the final volume of culture medium and DMSO concentration. The open question is then what is the required maximum test concentration in order to accept a negative test result, especially for those dispersions, which were delivered without clear indication of the amount of solid material (tattoo inks and gold).

For gold, sufficient exposure is shown by luciferase induction and cytotoxicity, similarly also for silver. Thus, even if the gravimetric concentration is not fully defined or below the maximal test concentration of the SOP, these tests are clearly acceptable.

For the two carbon black tattoo inks, neither cytotoxicity nor luciferase induction was reached. However, with a final level of 400 ppm for the dispersion, the amount of solid material at the maximum test concentration may well be below 200 ppm. Thus, the negative prediction according to the prediction model can be considered more uncertain for these test items, because higher exposure could theoretically still lead to a signal.

However, the test would then not be applicable. As observation of a reduction in background, luciferase already occurred in the tested concentration range. That could be due to some suppression of the luciferase light signal due to the black pigments not washed away during the wash step after the incubation time and prior to cell lysis for luciferase measurement (tattoo inks are made to permanently stain biological tissue).

As with any testing (*in vivo* and *in vitro*) of mixtures, a negative result is more uncertain as compared to testing pure chemicals as the exposure to the possible sensitising component is not maximized. Yet it should be kept in mind that the application of KeratinoSens™ is not stand alone and more information/combination of information (DA, IATA) is needed for a final assessment 'sensitiser' or 'non-sensitiser'. One possible modification of the test protocol is the removal of the test item dispersion after four hours incubation followed by a post-exposure incubation. The results indicate that this is clearly a possibility and leads to equal rating of all tested nanomaterials. It is possible that sufficient particles adhere to the cells / have been taken up by the cells within the first 4 hours, so that the additional contact time does not lead to higher exposure / biological response. Furthermore, a majority of MNM might have settled and adhered within 4 hours hindering the removal by simple pipetting of the supernatant. This is clearly different from testing of low molecular weight chemicals (see comparison for cinnamic aldehyde in Figure 10).

Though, there is also no clear advantage of this approach, as the cytotoxicity data indicate that for both forms of titanium dioxide and for nickel(II)oxides, cellular exposure is very similar, and luciferase induction is similar for silver and gold: only cytotoxicity could be reduced for silver by this approach.

Overall, technically the protocol with the small modifications made for substance preparations and substance additions appear applicable. Performing the PrestoBlue™ assay in parallel to the MTT assay is recommended, since this homogenous assay (performed in the supernatant of the still intact cells) seems to be less prone to assay interference. (Note: The MTT assay is performed on the adherent cells at the end of the experiment, and residual particles sticking to the cells may thus more easily interfere with the assay).

Routine testing would need modifications of the SOP especially to clearly described optimal substance preparation prior to addition to the cells. Finally, the standard protocol was followed here as closely as possible, e.g., in terms of DMSO addition, maximal test concentration, incubation time, and serum level. Of course, it is well possible, that different test parameters would lead to equally good or better results, and all these parameters are thus debatable, as they had been developed for testing low molecular weight chemicals. The problem is that there is not a large number of nanomaterials with very clear-cut sensitisation potential available in order to optimize these parameters for predictivity, while they were actually optimised for predictivity when testing well-defined low molecular weight sensitisers and non-sensitisers (Emter et al. 2010). Thus, in absence of any possibility to do a predictivity- based parameter optimisation, it was decided to stick as closely as possible to the validated test parameters, even if in principle they must not by definition be also optimal for nanomaterials – it is just impossible to find out and hence sticking to the validated parameters makes most sense.

9 Recommendations for adaptation of OECD TG 442D (KeratinoSens™ test method)

The recommendations are based on the discussions from the two expert workshops held in December 2019 and December 2021 as well as the feedback from a consultation within the OECD skin sensitisation expert group were the results and recommendations have been presented in October 2021 and February 2022.

- **Dispersion of nanomaterials:** The dispersion protocol applied should be selected based on the type of nanomaterial assessed in order to assure integrity of the nanomaterial after dispersion. In this study, the applied protocol was the NANoREG dispersion protocol (NANoREG). The NANoREG dispersion protocol uses a wetting step with ethanol that could affect or destroy the coating of some nanomaterials. Following the discussion at the expert workshop the DeLoid et al. (2017) protocol was additionally mentioned as it was assumed that this protocol might be used for a broader range of nanomaterials.

- **Characterisation of nanomaterials:** at least TEM and DLS should be performed in exposure media to have an idea on what the material might look like when exposed to the cells. The information out of this study showed that further characterisation steps (e.g., Rauscher et al. (2019), Mech et al. (2020b), Mech et al. (2020a) and Mech et al. (2020c)) might be necessary on a case by case basis in order to get a better understanding of what is the exposure of MNM to the test system. (See also the third paragraph in page 43 for further information.)

- **Endotoxin measurements:** Assessing the presence of endotoxin before testing of MNM is generally useful, as it is well known, that MNM might be contaminated with endotoxin. Therefore, the endotoxin testing is regarded as relevant to avoid misinterpretation of results, e.g., immune safety results. The experts concluded on the second expert workshop to refer to different endotoxin tests depending on the nanomaterial to be tested.
    - ISO guideline for Endotoxin (ISO/DIS 11737-3 under development)
    - Different commercially available Limulus amebocyte lysate (LAL) based assays
    - If nanomaterials interfere with the read-out of standard tests, an alternative could be an endotoxin determination based on analytical fatty acids measurements

  However, it has to be noted that endotoxin does not influence the results generated with KeratinoSens™, as this assay is not sensitive for endotoxin, because the Nrf2-Keap1-ARE toxicity pathway is not induced via activation of a toll like receptor 4 (TLR4) (Yin und Cao 2015).

- **Viability assay**: Use of a PrestoBlue™ assay in parallel to MTT is recommended, because the MTT assay might lead to solubility issues and nanomaterials might interfere with the absorption read-out of the formazan product.

- **Exposure time:** Exposure time should be kept at 48 hours. However, in case of aggregation of the nanomaterial or interference issues due to precipitates, a shorter nanomaterial exposure period (e.g., 4 hours) could be selected, depending on the cytotoxicity of these nanomaterials. The termination of the KeratinoSens™ assay would remain unchanged at 48 hours.

- **Leachates**: The use and application of nanomaterial leachates is seen as an optional step. It could be performed to check if the sensitising effect might be caused by the free ions or monomers.

- **DMSO**: The use of the solvent DMSO in the KeratinoSens™ assay might affect the nanomaterial uptake. It has been shown that low concentrations of DMSO (< 1 %) could already enhance the uptake of the nanomaterial into the cells (Gironi et al. 2020). Permeation enhancers, however, are common in sectors like cosmetics, where for the past two decades research has been focusing on the development of chemical components able to overcome the stratum corneum. Vehicles in the form of gels, emulsion or vesicle delivery systems have shown the potential to be effective for transdermal delivery (Kim et al. 2020a). Therefore, one could also argue that 1 % DMSO in KeratinoSens™ testing would reflect a worst-case scenario. Further, in this study positively tested MNM were additionally tested in KeratinoSens™ without DMSO. No difference in the outcome of results has been identified for silver and pigment red 170 tattoo ink (Figure 12 and Figure 13 in Annexe). The outcome of gold was positive but the signal was much weaker compared to the testing in KeratinoSens™ with DMSO (Figure 14 in Annexe), same has been shown with the positive control substance of cynnamic aldehyde (Figure 15 in Annexe).
  Therefore, a final conclusion about DMSO as a penetration enhancer for the tested MNM could not be made. However these data show that DMSO increases the sensitivity of KeratinoSens™, which has also been observed during assay development and therefore included in the validated protocol (OECD 2018). Hence, it should always be used.

- **Animal / Human serum**: KeratinoSens™ is routinely run with 1% fetal bovine serum and since the cells are not immunocompetent no effects due to the species difference for the serum used is known. However, an adaptation using human serum has been added to OECD TG 442D (KeratinoSens™ test method), and this could also be used for nanoparticle testing as an option.

10 Limitations of this study

The recommendations on the technical applicability of OECD TG 442D (KeratinoSens™ test method) in this study report are derived from data obtained from a small number of MNM performed in a single laboratory having expertise in routinely testing chemicals with KeratinoSens™. They are, however, further based on consultation with experts from the field of nanomaterials and skin sensitisation, some of whom had made similar experience in testing MNM in other skin sensitisation test methods. As *in vivo* data in literature on skin sensitisation and MNM was scarce, only a limited qualitative assessment of the *in vitro* results to *in vivo* information from literature could be performed. These initial comparison is a good starting point, any further issues related to the potential use of OECD TG 442D (KeratinoSens™ test method) for MNM in a regulatory context will of course have to be addressed in a much broader context (DA, IATA).
In future also the discussion about the accepted criteria of MNM exposure to the test system (e.g., agglomeration/aggregation state) needs to be discussed. This would not be limited to MNM testing for skin sensitisation potential but include general MNM testing.

11 Annexe

Detailed results Physico-chemical data comparison between published and selected materials

Following the extensive literature review described previously in this study report, MNM were organised/ordered with best physico-chemical characteristics overlap to the ones referenced in literature. In some cases, we were unable to contact the authors of the publications, i.e., regarding zinc and titanium on UV blocking textiles (see above), and a relevant pristine material was purchased. In the case of the TiO_2 used in textiles, due to the titanium being amino-functionalised, we could unfortunately not identify a matching counterpart to test the system, as the amino group potentially may have a role as a sensitiser. In other cases, we managed to get in touch with the suppliers who sent us the relevant material (e.g., Sukgyung AT) or indicated that the product had been discontinued (e.g., ABC Nanotech Co. and silver). Again, when the product was no longer available, the material closest resembling the description provided in the publication was purchased.
Available information on the physico-chemical parameters from the literature of the selected MNM are compared in Table 11 with measured values from this study.

Table 11 Size comparison between those MNM identified in the literature and the ones selected to be tested within this study (highlighted in green).

Sample	TEM magnification (X)	Feret-size					
		Median diameter	Mean diameter		Mode diameter	Range	
		nm	nm	SD (nm)	nm	Min (nm)	Max (nm)
Nickel(II)oxide	160000	22.5	34.5	35.3	24.3	4	264
Nickel metal Literature	-	-	20				
Titanium(IV)oxide	43000	46.8	61.6	51.3	32.9	10	371
Titanium(IV) literature			<25 nm diameter				
Carbon black (Pitch Black)	20500	195.3	224.7	142.6	158.1	37	795
Carbon black (Pitch Black) literature							
Carbon black (True Black)	9900	327.1	360.4	258.1	196.3	65.4	1654.2
Carbon black (True Black) literature							
Silver	300000	6.7	7.1	2.2	5.2	1	12
Silver literature			10				
SG-TO50 (TiO_2)	16500	91.4	168.3	172.1	51.4	11	1000
Pigment red 170	20500	168.2	231.8	249.6	18.7	9.3	1392.5
Pigment red 170 literature							
Gold	220000	5.8	6.0	1.0	5.8	1	9
Gold literature			5				

Highlighted in orange, no comparable TEM value was provided by the supplier of SG-TO50 (TiO_2).

As seen on the table, very little information regarding physico-chemical parameters was obtained from those particles identified in the literature.

Measured KeratinoSens™ values

I_{Max} values of tested nanomaterials

Table 12 and Table 13 show the measured I_{Max} values for tested nanomaterials and positive control substances encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors.
I_{Max} values for Titanium(IV)oxide, nickel(II)oxide and, SG-TO50 (TiO_2) indicate maximal fold-induction up to a concentration of 256 µg/mL, whereas I_{Max} values of gold, silver and tattoo inks indicate maximal fold-induction up to the maximal test concentration and the I_{Max} values of positive control substances indicate maximal fold-induction up to a concentration of 200 µg/mL.

Table 12 I_{Max} values of tested nanomaterials within KeratinoSens™

Test substance	Rep 1 I_{Max} (fold induction)	Rep 2 I_{Max} (fold induction)	Rep 3 I_{Max} (fold induction)	Average I_{Max} (fold induction)	Standard deviation I_{Max}
SG-TO50 (TiO_2)	1.48	1.41	1.14	1.34	0.18
Titanium(IV)oxide	1.50	1.43	1.40	1.44	0.05
Nickel(II)oxide	1.30	1.08	0.86	1.08	0.22
Silver	266.43	268.91	95.17	210.17	99.60
Gold	3.90	5.21	2.61	3.91	1.3
Carbon black (True Black)	1.31	1.70	1.21	1.41	0.26
Carbon black (Pitch Black)	1.37	1.71	1.17	1.42	0.27
Pigment red 170 tattoo ink	2.41	3.38	1.94	2.58	0.74

Table 13 I_{Max} values for positive control substances and positive control substances encapsulated in poly-ε-caprolactone nanoparticles (NP) vectors.

Test substance	Rep 1 I_{Max} (fold induction)	Rep 2 I_{Max} (fold induction)	Rep 3 I_{Max} (fold induction)	Average I_{Max} (fold induction)	Standard deviation I_{Max}
Citral encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	46.48	28.05	53.07	42.53	10.59
Citral	70.51	23.49	29.19	41.06	20.95
Cinnamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	22.03	8.53	9.23	13.26	6.20
Cinnamic aldehyde	13.10	8.80	14.47	12.12	2.41
Isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	25.64	28.67	20.35	24.89	3.44

Isoeugenol	104.44	80.88	74.15	86.49	12.98

EC1.5 value of tested nanomaterials and positive control substances encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors

Table 14 describes the EC1.5 value of SG-TO50 (TiO2), titanium(IV)oxide, and nickel(II)oxide as the concentration in µg/mL inducing the luciferase activity 1.5-fold up to a concentration of 256 µg/mL and the EC1.5 value of the tested tattoo inks as the concentration in µg/mL inducing the luciferase activity 1.5-fold up to a concentration of 200 µg/mL, whereas the EC1.5 value of gold and silver as the concentration in µg/mL inducing the luciferase activity 1.5-fold up to the maximal test concentration are shown.

Table 14 Luciferase determination of tested nanomaterials and positive control substances (EC1.5)

Test substance	Rep 1 (µg/mL)	Rep 2 (µg/mL)	Rep 3 (µg/mL)	Geometric Mean
SG-TO50 (TiO_2)	n.i.	n.i.	n.i.	n.i.
Titanium(IV)oxide	n.i.	n.i.	n.i.	n.i.
Nickel(II)oxide	n.i.	n.i.	n.i.	n.i.
Silver	794.86 [1)]	395.82	30433.37	2123.48
Gold	25.54 [1)]	< 61	120.42	57
Carbon black (True Black)	n.i.	1.25	n.i.	n.i.
Carbon black (Pitch Black)	n.i.	0.54 [1)]	n.i.	n.i.
Pigment red 170 tattoo ink	18.33 [1)]	0.42	39.49	6.70
Citral encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	2.96	2.17	2.14	2.40
Citral	0.94	1.62	3.22	1.70
Cinnamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	1.12	2.39	2.26	1.82
Cinnamic aldehyde	1.43	2.26	2.30	1.95
Isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors	1.04	1.80	1.06	1.25
Isoeugenol	0.95	1.94	2.09	1.57

[1)] Here the tested concentration of the particles dispersion as obtained is given and not the final concentration of the particles (which is not exactly known).

Numerical results for the positive control cinnamic aldehyde

Cinnamic aldehyde needs to be positive for a run to be accepted (i.e., induction > 1.5 fold). This requirement was fulfilled in all three repetitions of all three experiments.
The induction at 64 µM and the EC1.5 for cinnamic aldehyde were also calculated. The targets are: (i) Average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8, and (ii) the EC1.5 value should be between 7 µM and 30 µM. At least one of these two numerical criteria must be met in order to accept a repetition. Both criteria were fulfilled in 7 of the 9 runs.
In repetition 3 of the first series, the EC1.5 value for cinnamic aldehyde was within the range, but the average induction at 64 µM was below 2. As one criterion was fulfilled, the run was still valid.
In repetition 1 of the third series, the EC1.5 value for cinnamic aldehyde was below the target range, but the average induction at 64 µM was between 2 and 8. As one criterion was fulfilled, the run was still valid.
As second performance criterion, the variability of the solvent control must be below 20%. Table 15 lists the results of all three repetitions in all three experimental series. All nine runs were valid for the solvent control.

Table 15 Numerical results for the positive control cinnamic aldehyde

Quality control: Induction values Reference cinnamic aldehyde							Criteria fullfilled		Quality control	
	4 µM	8 µM	16 µM	32 µM	64 µM	EC 1.5	EC1.5	Ind. 64 uM	% standard deviation blanks	
Testing of powders dispersed with ultrasonication										
rep1	1.1	1.3	1.9	2.1	3.9	10.4	TRUE	TRUE	12.8	ACCEPTED
rep2	1.1	1.3	1.4	1.8	2.4	19.5	TRUE	TRUE	11.3	ACCEPTED
rep3	1.0	1.0	1.3	1.6	1.6	28.6	TRUE	FALSE	17.9	ACCEPTED
Testing of Dispersions (silver, gold, tattoo inks)										
rep1	1.4	1.4	1.4	2.0	2.1	18.6	TRUE	TRUE	16.4	ACCEPTED
rep2	1.2	1.4	1.5	1.7	2.1	12.9	TRUE	TRUE	15.5	ACCEPTED
rep3	1.2	1.4	1.5	1.9	2.7	13.3	TRUE	TRUE	12.7	ACCEPTED
Testing of caprolactone samples										
rep1	1.3	1.6	2.0	2.6	4.4	6.9	FALSE	TRUE	13.0	ACCEPTED
rep2	1.2	1.3	1.6	1.9	3.0	13.8	TRUE	TRUE	8.8	ACCEPTED
rep3	1.1	1.4	1.6	1.9	2.7	11.2	TRUE	TRUE	3.5	ACCEPTED

Table 16 Historical runs of KeratinoSens™

Historical runs conducted between 2010 and 2021

Historical runs (n= 623)						
	4 µM	8 µM	16 µM	32 µM	64 µM	EC1.5
Average	1.2	1.3	1.5	1.9	3.3	16.4
StDev	0.1	0.14	0.2	0.35	2.6	6.9

Results of performed KeratinoSens™ testing without DMSO

As previously stated, DMSO can have an influence of the uptake of MNM by the cells (Gironi et al. 2020). Therefore, additional testing of the positively tested MNM silver, gold and pigment red 170 tattoo ink as well as the positive control substance cinnamic aldehyde without DMSO has been conducted. The outcome of the testing remained very similar for silver (Figure 12) and pigment red 170 tattoo ink (Figure

13) with and without DMSO. But the sensitivity of the KeratinoSens™ was reduced when DMSO was missing, results of gold (Figure 14) and cinnamic aldehyde (Figure 15) testing revealed a lesser intense positive signal after KeratinoSens™ testing.

Figure 12 Silver tested in KeratinoSens™ without DMSO (left) and with DMSO (right)

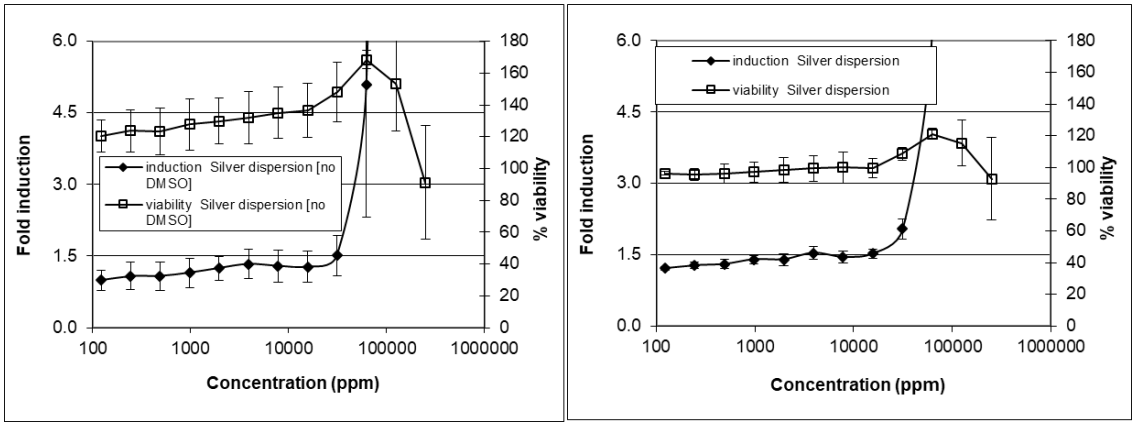


Figure 13 Pigment red 170 tattoo ink tested in KeratinoSens™ without DMSO (left) and with DMSO (right)

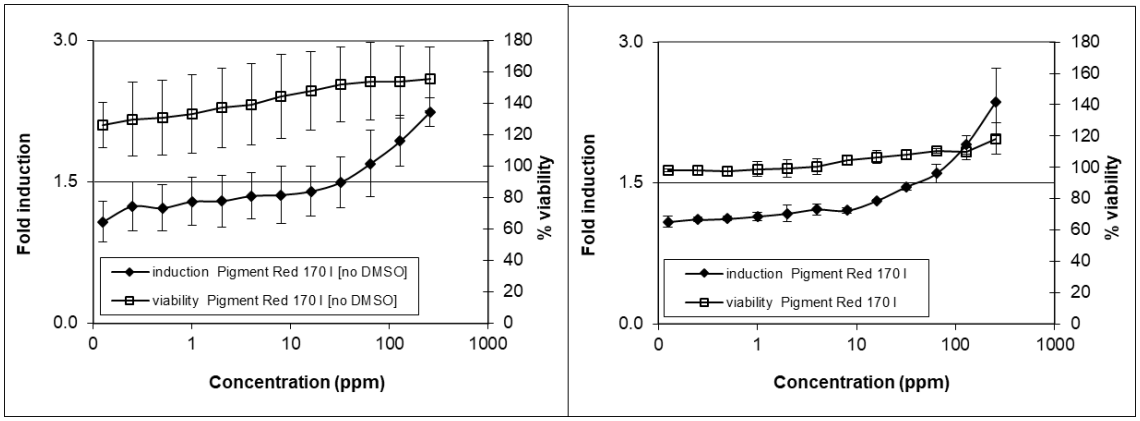


Figure 14 Gold tested in KeratinoSens™ without DMSO (left) and with DMSO (right)

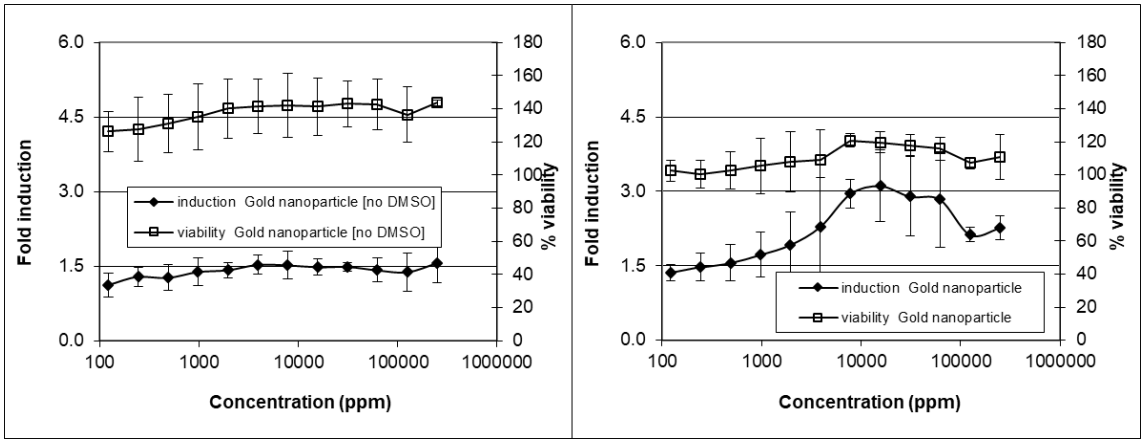
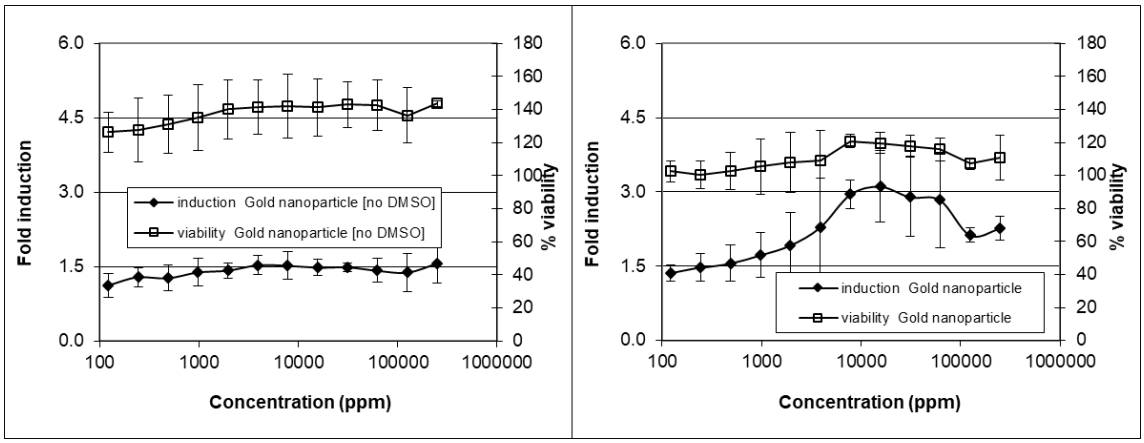


Figure 15 Cinnamic aldehyde tested in KeratinoSens™ without DMSO (left) and with DMSO (right)



Overview of literature studies used for in vitro to in vivo comparison

Several publications, Opinions and Reports were reviewed to identify relevant nanomaterials as reported in the section above "Proposed referenced substances". To further understand the source of the *in vivo* data and the particulars of each selected material, a summary of the selected cases is provided below:

- Lymph node proliferation assay (LNPA) (Auttachoat et al. 2014): Generally the local lymph node assay (LLNA) is used to predict the immunotoxicity of small molecules in human. This assay is based on the topical exposure of the chemicals to the skin. MNM, however, may have a different route of entry into the body, which also depends on their physicochemical characteristics. To avoid uncertainties regarding final exposure concentrations, a modification of the LLNA assay, the LNPA is generally recommended for MNM, since this method is based on exposure through subcutaneous injection (Dobrovolskaia et al. 2009). In the study reported by Auttachoat et al. (2014), 0.1 mL TiO_2 (anatase; 525 nm diameter) was administered subcutaneously along the mid-line on top of the head of eight female BALB/c mice. Dose range covered 12.5, 37.5, 125, and 250 mg/kg. Dispersions were prepared daily and stirred for 24 hours to achieve homogeneity. Methylcellulose (MTC; 0.5% w/v; CAS No.9004-67-5) was used as vehicle. As a positive control 2,4-dinitrofluorobenzene (DNFB; CAS No. 70-34-8) was used at 0.1% (v/v) in 0.5% methylcellulose, at a total volume of 100 mL/mouse (0.1% approximately 30 mg/kg). Identical treatments were repeated for the next 2 days. The mice were rested on days 4 and 5. On day 6, mice were injected intravenously with 20 mCi [3H]-thymidine and evaluated for lymph node cell proliferation. As a result, lymph node proliferation in mice exposed to 125 mg/kg exceeded the 3-fold threshold of the vehicle control response. At the 250 mg/kg dose, a statistically significant increase was also observed; however, the 3-fold threshold was not achieved.

- Occupational health case report (Journeay und Goldman 2014). This case was reported from a company producing metallic inks. The patient was a 26-year-old non-smoking female, generally working in this company as a polymer formulation chemist. Her job changed and she was asked to measure nano metal nickel powder (1-2 g). The nickel metal particles were 99% pure, round particles with a surface area of 40-60 m^2/g and an aerodynamic particle size of 20 nm. She was also involved in handling the downstream process with nano metal nickel. The work was performed on an open laboratory bench wearing protective latex gloves. A week later she started suffering from allergic rhinitis. A T.R.U.E patch test showed a positive reaction to nickel. She was moved to another laboratory within the same company with no metal chemistry job and her symptoms improved.

- Gold nanoparticles as Nrf2 activators (Goldstein et al. 2016): This publication reports that macrophages and keratinocytes exposed to 5 nm gold nanoparticles (NPs) at a concentration of 9 nM

were able to induce Nrf2 translocation to the nucleus, suggesting a possible activation of the Nrf2–Keap1 pathway by gold NPs.

- Skin sensitisation evaluation of silver NPs (Kim et al. 2013): a toxicity evaluation including skin sensitisation was performed using 10 nm silver NPs (nanosized colloidal silver at 28.48% concentration then dispersed in 1% citric acid). Skin sensitisation was performed following OECD TG 406 on male guinea pigs and on three different days. On the first day animals were exposed to 0.1 mL of colloidal silver, on day 5 the test area was painted with 0.5 mL of 10% sodium dodecyl sulphate in vaseline before applying the topical induction. The following day (day 6), a filter paper loaded with 0.5 mL of silver (102.4 mg) in a vehicle (1% citrate solution) was then applied to the test area and held in contact by an occlusive dressing for 48 hours. A challenge test was also conducted 21 days after the initial injection of the test substance with either nanosilver or vehicle. 48 hours from the start of the challenge application, the skin reaction was observed and recorded according to the Magnusson/Kligman grading scale (Magnusson und Kligman 1969). Approximately 24 hours after this observation, a second observation (72 hours) was made and once again recorded. At 24 and 48 hours after removing the challenge patch, one animal (1/20) exhibited discrete and patchy erythema. The skin sensitisation rate for the silver NPs was determined as 5% (1/20) and the test substance categorised as a class I 'weak' skin sensitizer.

- OECD Series on Manufactured Nanomaterials: Titanium Dioxide (OECD Environment Directorate 2016f): In 2007 the OECD Working Party on Manufactured Nanomaterials (WPMN) launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (Testing Programme) to evaluate if current testing protocols were suitable to nanomaterials. The Testing Programme focused on the 11 most industrially relevant materials and results have been published free of charge and can be downloaded from the OECD website[4] . During the revision of the 11 dossiers within this study the positive entry for skin sensitisation for TiO_2 dedicated dossier has been noted. The material itself was called Sukgyung (SG-TO50) and the manufacturers provided no information on its physico-chemical characteristics. The material was tested on guinea pigs following OECD TG 406 but no dosing information was provided. Results showed that 1/17 exhibited grade 1 erythema and the material was regarded as a weak/ambiguous sensitizer (not fulfilling the classification criteria for skin sensitisation).

- Tattoo inks: tattoo inks have been associated with skin sensitisation, even though little research on their toxicology has been performed. Generally, tattoo inks manufacturers do not disclose the exact content of their products, which makes it difficult to assess where toxicity comes from. From January 2022, however, thousands of hazardous chemicals found in tattoo inks and permanent make-up are restricted in the EU under the REACH Regulation[5] . A recent opinion from the German Institute of risk assessment included the skin sensitisation approach based on IATAs on its risk assessment strategies for tattoo inks (Bundesinstitut für Risikobewertung 2021). Several studies have also associated tattoo inks with allergic reactions, with red being the most problematic colour, and with sensitisation induction coming up months or years later (Laux et al. 2016). These complications can be linked to individuals being exposed to potential allergens continuously during their lives. Currently there is an absence of reliable experimental approaches to test tattoo inks and therefore we included them in this study, based also on previous reports on the presence of nanomaterials in such inks (Grant et al. 2015). In this study we have selected a widely used colour such as black and the most recurrent colour producing complications, e.g. red (Laux et al. 2016).

[4] Testing Programme of Manufactured Nanomaterials - OECD (last assessed March 2022)

[5] Tattoo inks and permanent make-up - ECHA (europa.eu) (last accessed March 2022)

Table 17 Regulatory requirements related to skin sensitisation

Region/Country	Chemical sector	Endpoint	Accepted *in vivo*	*In vitro* strategies
Brazil	Cosmetic/personal care	Hazard	Clinical studies, LLNA and Buehler, GPMT	Yes, as part of an integrated strategy
	Pesticides and plant protection products	Hazard	LLNA and Buehler, GPMT	Yes, as part of an integrated strategy
	Pharmaceuticals		Clinical studies, LLNA and Buehler, GPMT	Yes, as part of an integrated strategy
Canada	Cosmetics	Risk	Submitted on request. Open literature accepted	Yes
	Household products/art materials	Risk	LLNA, GPMT, Buehler	Yes, if animal data are unavailable
	Industrial chemicals (on Domestic Substances list)	Potency/risk	Data submission not required (LLNA, GPMT, Buehler accepted)	Yes (in voluntary submission)
	Chemicals not listed on Domestic Substances list	Potency/risk	LLNA, GPMT, Buehler accepted	Considered on a case-by-case basis
	Medical Devices	Hazard	GPMT, LLNA	Yes, if validated
	Pesticides	Hazard	LLNA, GPMT, Buehler	Yes, as part of an integral strategy
	Prescription Pharmaceuticals	Hazard, risk	Not specified	Yes, with justification
	Topical Non-prescription Pharmaceuticals	Hazard	LLNA; GPMT, Buehler, Clinical studies	Yes, with justification
	Work place chemicals	Hazard, potency	New testing not required	No
European Union	Biocides	Hazard	LLNA	Yes
	Cosmetics	Hazard, potency, risk	Banned	Yes
	Household Chemicals	Hazard, potency, risk	LLNA	Yes
	Industrial Chemicals	Hazard, potency, risk	LLNA	Yes
	Pharmaceuticals	Hazard	Not specified	Yes
	Plant Protection products	Hazard	LLNA	No
	Workplace Chemicals	Hazard, potency, risk	LLNA	Yes
Japan	Cosmetics and Personal Care products	Hazard, potency	GPMT, Buehler, LLNA	Yes

Glossary

AKR1C2	Aldo-keto reductase family 1, member C2
ARE	Antioxidant response element
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
DA	Defined Approach
C.I.	Colour index
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EC1.5	Extrapolated concentration for a 1.5 fold luciferase induction
EU	European Union
FCS	Fetal Calf Serum
GHS	Globally Harmonized System for the Classification and Labelling of Chemicals
GPMT	Guinea Pig Maximization Test
HaCaT	Human Keratinocyte Line
IATA	Integrated Approaches to Testing and Assessment
IC50	Concentration for reduction of cellular viability by 50%
I_{Max}	Maximal induction of luciferase activity over solvent control over the complete dose response range measured
Keap1	Kelch-like ECH-associated protein 1
LAL	Limulus Amebocyte Lysate based assay
LLNA	Local Lymph Node Assay
LNPA	Lymph nOde Proliferation Assay
MNM	Manufactured Nanomaterials
MTT	Thiazolyl Blue Tetrazolium bromide
MW	Molecular Weight
NPs	Nanoparticles
Nrf2	Nuclear factor (Erythroid-derived 2)-like 2
OECD	Organisation for Economic Co-operation and Development
PAHs	polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PDI	Polydispersity index
PEG	Polyethylene Glycol
pH	pondus Hydrogenii
PPM	Parts Per Million
REACH	EU Chemicals Regulation “Registration, Evaluation, Authorisation and Restriction of Chemicals”
SOP	Standard Operation Protocol
TEM	Trans Electron Microscopy
TG	Test Guideline
TiO_2	Titanium dioxide
TRL4	Toll Like Receptor 4

WNT	Working Group of National Co-ordinators if the Test Guidelines Programme
WPMN	Working Party on Manufactured Nanomaterials

Technical information of tested nanomaterials

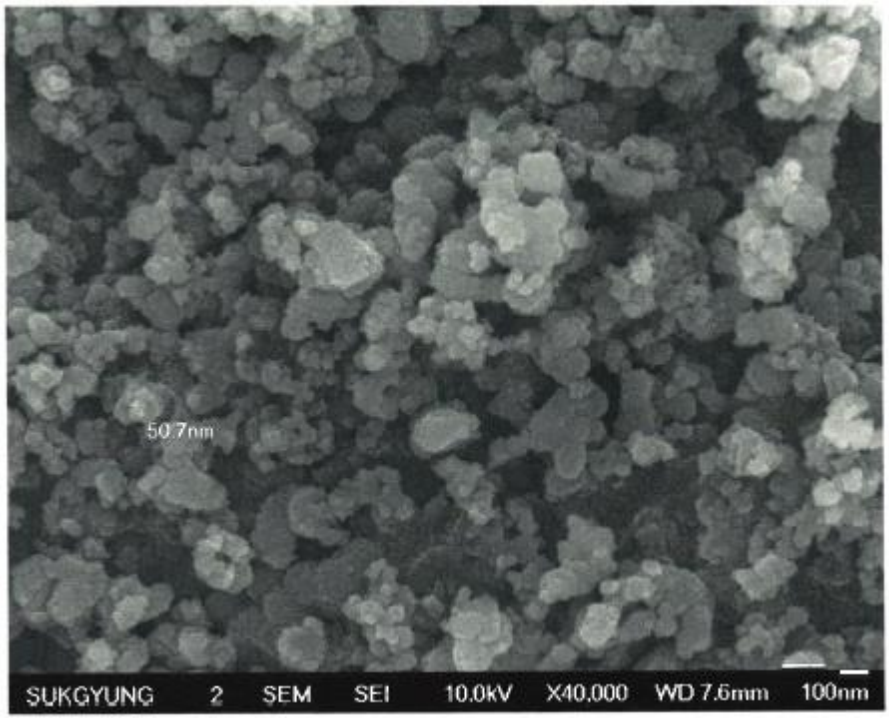
The following technical information were provided by the producers of the purchased MNM, these MNM were tested in this project.

SG-TO50 (TiO2)

Property	Specification	Result	Comments
Appearance	White powder		Ti02
Particle Size (nm)	50 ± 10	50.7	JSM 6701F (JEOL)
Whiteness	> 70	88.38	CM-3600d (MINOLTA)
Moisture Contents (Yo) (105°C, 1 hour)	< 0.5	0.25	Drying Oven

Manufacture Date: 2019.04.12
Shelf Life: 5 years

Figure 16 FE-SEM picture of SG-TO50

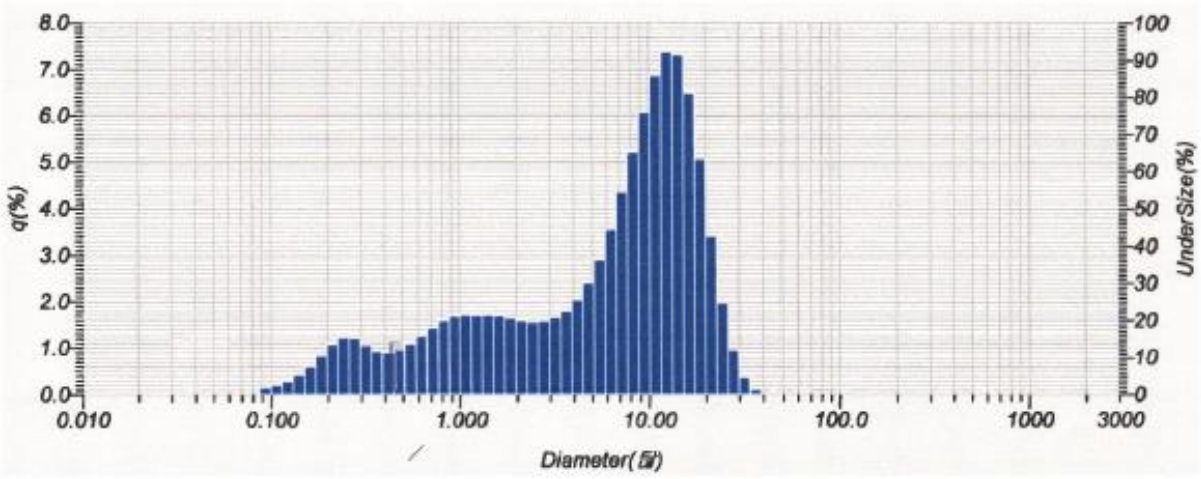


HORI BA Laser Scattering Particle Size Distribution Analyzer LA-950:

Sample name	SG-TO50	Median Size	7.94462 (µm)
Lot number	SG-UHNWUZA4	Mean size	8.65544 (µm)
Transmittance(R)	94.8 (%)	Std. Dev.	7.0030 (µm)
Transmittance(B)	88.6 (%)	Geo. Mean Size	4.7571 (µm)
Circulation Speed	5	Geo. Std. Dev.	3.9410 (µm)
Agitation speed	5	Mode Size	12.4288 (µm)

Form of Distribution	Auto	Span	OFF	
Distribution Base	Volume	Diameter on Cumulative %	(2)10.00 0.5198 (µm)	(%)-
Refractive Index (R)	TiO2(W)[Tio2(W)(2.500-0.00i),Water(1.333)]		(5)50.00 7.9446 (µm)	(%)-
Refractive Index (B)	TiO2(W)[Tio2(W)(2.500-0.00i),Water(1.333)]		(9)90.00 18.2782 (µm)	(%)-

Figure 17 Laser Scattering particle size distribution of SG-TO50



Titanium(IV)oxide

Product Specification
Product Name: Titanium(IV)oxide, anatase – nanopowder, <25 nm particle size, 99.7% trace metal basis

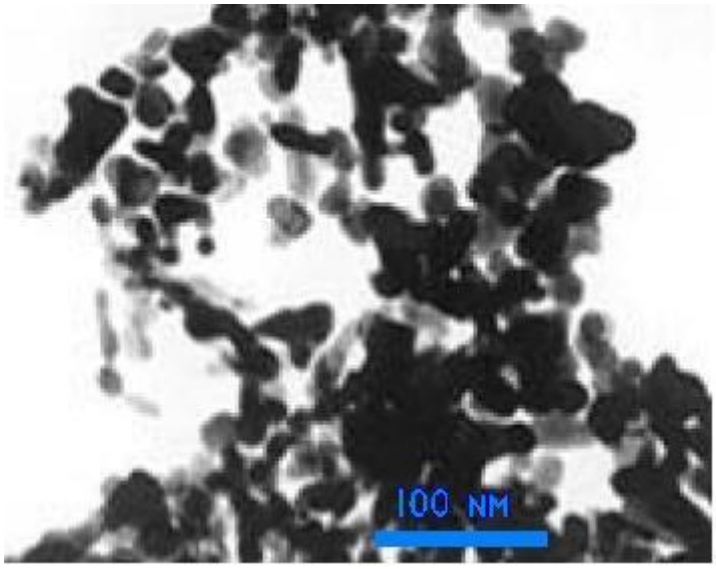
CAS Number:	1317-70-0	
MDL:	MFCD00011269	
Formula:	O2Ti	
Formula Weight:	79.87 g/mol	
Appearance (Color)		White
Appearance (Form)		Powder
X-Ray Diffraction		Conforms to Structure
Particle Size		<_ 25 nm
Surface Area		45 - 55 m2/g
ICP Major Analysis		Confirmed
Confirms Titanium Component		
Purity	99.7% Based On Trace	Meets Requirements
Metals Analysis		
Trace Metal Analysis		<_ 3500.0 ppm

Nickel(II)oxide

Composition / Information on ingredients

Ingredient name	Nickel(II)oxide
CAS No.	1313-99-1
Empirical Formula	NiO
Molecular weight	74.69 amu
Purity	99.5%
Average Particle Size	20 nm
Specific Surface Area	> 50 m^2/g (BET)
Appearance	Black powder
Melting Point	1957 °C (+/- 20°C)
Density	6.7 g/cm^3

Figure 18 TEM image of nickel(II)oxide



Silver

Product Specification
Product Name: Silver, dispersion - nanoparticles, 10 nm particle size (TEM), 0.02 mg/mL in aqueous buffer, contains sodium citrate as stabilizer
Formula: Ag
Weight: 107.87 g/mol
Storage temperature: 2- 8 ℃

TEST	Specification
Appearance (Colour)	Faint Yellow to Dark Yellow and Faint Orange to Dark Orange
Appearance (Form)	May Appear Hazy Due to Being A Liquid

Dispersion	
ICP Major Analysis	Confirmed
Confirms Silver Component	
Wavelength	380 - 405 nm
Absorbance	0.92 - 1.23 Abs UN

Gold

Product Specification

Product Name: Gold nanoparticles – 5 nm diameter, OD 1, stabilized suspension in citrate buffer

Formula:	Au
Formula Weight:	196.97 g/mol
Storage Temperature:	2 - 8 °C

TEST	Specification
Appearance (Form)	Suspension
Polydispersity Index (PDI)	< 0.2_
Core Size	4 - 7 nm
Mean Hydrodynamic Diameter (Z)	14 - 25 nm
Particles/ml	Confirmed
4.92E+13-6.01E+13	
Absorption Max	510 - 525 nm

Carbon black True Black tattoo ink

Composition / Ingredients

Name/ C.I. Number	**C.A.S. #**	**EC #**
Aqua	7732-18-5	231-791-2
C.I.77266	1333-86-4	215-609-9
Glycerine	56-81-5	200-289-5
Isopropyl Alcohol	67-63-0	200-661-7
Hamamelis Virginiana	84696-19-5	283-637-9

Chemical and physical property

Physical state:	Liquid
Odor:	Faint odour of ammonia
pH:	2.0 to 6.0
Stability:	Stable under ordinary conditions of temperature and usage

Carbon black Pitch Black tattoo ink

No technical information was provided by the supplier of carbon black Pitch Black tattoo ink.

Pigment red 170 tattoo ink

Identification of the substance

Identification of the substance	Pigment red 170

Additional identification Pigment red 170 F5RK

Substance related information

Substance name	Pigment red 170
Synonymes	Pigment red 170 F5RK
Cas No.	[2786-76-7]
Formula	$C_{26}H_{22}N_4O_4$

Physical and chemical properties
Colour: Red
Form: Powder
Molar Mass: 454.48 g/mol
Stability and reactivity
Conditions to Avoid: incompatible materials
Substances to Avoid: strong oxidizers
Decomposition Products: nitrogen oxides (NOx), carbon dioxide (CO2), carbon monoxide

Polyethylenglycoldiacrylate nanotubes (PEGDA 575)

Detailed information on PEGDA 575 is available in the paper by Newland et al. (2018) and the additional supportive information.

Citral encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors

Batch: 202009-citr-01.
1.10 of product.
Composition of the solution:

- Water: ~185 mL
- Polycaprolactone (14 kDa): 0 5 mg/mL
- Polysorbate 80: 0.25 mg/mL
- Citra! (natural): 5.2 mg/mL
- 91 % of citral is encapsulated.
- pH = 4.8

Size
Determined by DLS in water.

- Hydrodynamic diameter: 139.6 nm
- Standard deviation: 43.9
- Polydispersity: 0.066

Zeta potential
Measured after dilution in NaCI 0.1%

- Value: -3.59 mV
- Standard deviation: 5.77

Cinnamic aldehyde encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors

Batch: 202009-cinn-01.
1.36 g of product.
Composition of the solution:

- Water: ~185 mL
- Polycaprolactone (14 kDa): 0.5 mg mL
- Polysotbate 80: 0.25 rng/mL.

- Cinnamaldehyde (natural): 6.6 mg/mL
- 80 % of cinnamaldehyde is encapsulated
- pH = 4.2

Size

- Determined by DLS, in water.
- Hydrodynamic diameter: 148.7 nm.
- Standard deviation: 39.5
- Polydispersity: 0.028

Zeta potential

Measured after dilution in NaCl 0.1%

- Value: -3.16 mV
- Standard deviation: 5.58.

Isoeugenol encapsulated in poly-ε-caprolactone nanoparticle (NP) vectors

Batch: 202009-iso-01

1.24 g of product

Composition of the solution:

- Water: ~185 ml.
- Polycaprolactone (14 kDa): 1 mg/mL
- Polysorbate 80: 0.5 mg/mL
- Isoeugenol (natural): 5.2 mg/mL
- 86 % of isoeugenol is encapsulated
- pH = 4.8.

Size

Determined by DLS, in water.

- Hydrodynamic diameter: 147.2 nm
- Standard deviation: 41.6.
- Polydispersity: 0.054

Zeta potential

Measured after dilution in NaCl 0.1 %

- Value: -2.70 mV.
- Standard deviation: 4.94.

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