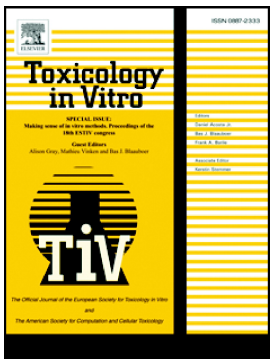

A comparison of dermal toxicity models; assessing suitability for safe(r)-by-design decision-making and for screening nanomaterial hazards

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A comparison of dermal toxicity models; assessing suitability for safe(r)-by-design decision-making and for screening nanomaterial hazards

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Abstract

The objective of Safe-by-Design (SbD) is to support the development of safer products and production processes, and enable safe use throughout a materials' life cycle; an intervention at an early stage of innovation can greatly benefit industry by reducing costs associated with the development of products later found to elicit harmful effects. Early hazard screening can support this process, and is needed for all of the expected nanomaterial exposure routes, including inhalation, ingestion and dermal. In this study, we compare *in vitro* and *ex vivo* cell models that represent dermal exposures (including HaCaT cells, primary keratinocytes, and reconstructed human epidermis (RhE)), and when possible consider these in the context of regulatory accepted OECD TG for *in vitro* dermal irritation. Various benchmark nanomaterials were used to assess markers of cell stress in each cell model. In addition, we evaluated different dosing strategies that have been used when applying the OECD TG for dermal irritation in assessment of nanomaterials, and how inconsistencies in the approach used can have considerable impact of the conclusions made. Although we could not demonstrate alignment of all models used, there was an indication that the simpler *in vitro* cell model aligned more closely with RhE tissue than *ex vivo* primary keratinocytes, supporting the use of HaCaT cells for screening of dermal toxicity of nanomaterials and in early-stage SbD decision-making.

Keywords: nanomaterial; safe-by-design; hazard screening; dermal toxicity; new approach methodologies (NAMs).

Introduction

Nanomaterials (NMs) are increasingly appearing in consumer products, most notably in cosmetic and biomedical products (e.g. sunscreens, foundations (Dreno et al., 2019), and wound healing products (de Souza et al., 2021). Many of these products are intended for skin application, therefore of particular concern is the hazard these materials may pose following sustained dermal exposure. Traditional dermal hazard assessment investigates the short-term effect to skin after exposure, namely irritation and corrosion, whereby in vitro test methods have been successfully validated and are available through the OECD (OECD, 2020, OECD, 2019). Although useful, these modes of toxicity may not always be relevant to NMs, and other particulates, that are applied to the skin and may be retained for long periods of time. In fact, it is believed that nanoparticles (NPs) may be retained in the hair follicles for up to 10 days following initial exposure (Lademann et al., 2007), therefore far longer exposure times may be more relevant for NMs than bulk or liquid chemicals. Solid particles, and in particular NMs, have a range of possible toxicity mechanisms, driven by their physico-chemical properties, such as dissolution rate and surface reactivity. Therefore, the methods used to assess dermal hazard should consider these mechanisms of toxicity of NMs.

Moreover, as defined by Ruijter et al. (2023) the emerging focus on safe(r)-by-design (SbD) principles leads us into the need to provide cost-effective, high-throughput methods, with a high degree of sensitivity and an ability to predict more recognised/accepted methodology. With this in mind, the current study aims to compare the output of different dermal testing models, identify when assay optimisation can improve correlation, and to provide guidance of when different models are most useful for SbD decisions. We have compared different dermal testing models; these include 2D models consisting of either a stable keratinocyte cell line (HaCaT cells) or primary keratinocytes, and a more complex reconstituted human epiderm (RhE) tissue. These models were optimised to allow comparisons to OECD guidance, and expanded for more general assessment of dermal toxicity, as to align with screening processes to support SbD. The keratinocyte cell line HaCaT are easy to culture and have been suggested previously as a suitable *in vitro* model for initial screening of dermal irritation (Gibbs, 2009). It follows similar work, in which a 2D HaCaT model has been shown to be predictive of *in vivo* dermal sensitisation for chemicals (Chung et al., 2018, Jeon et al., 2019); we have expanded on this work to consider how a similar model for dermal toxicity can be defined for NMs. It is hoped that, if successful, this dermal toxicity model could be incorporated into a tiered testing strategy (TTS), such as those used in tailored Integrated Approach to Testing and Assessments (IATAs) for the purposes of grouping NMs upon dermal exposure (Di Cristo et al., 2022). In addition, we evaluated the HaCaT cell model under the concept proposed by Chung et al. (2018) and Jeon et al. (2019), in which these cells are used as a model to identify skin sensitisation hazards.

We tested the HaCaT model with a range of benchmark NPs, including TiO_2, SiO_2, Ag, ZnO and CuO. The panel tested included ZnO and CuO NPs as possible dermal irritants; in the "Summary of Classification and Labelling" on the ECHA website, each chemical has notified classification and labelling as Skin Irritants (Category 2), however have not been identified as skin irritants in the CLP classification. There are also examples in literature of toxic effects of ZnO and CuO NPs to dermal models (Alarifi et al., 2013, Ge et al., 2017, Jang et al., 2012, Vinardell et al., 2017). TiO_2 and SiO_2 NPs were chosen as particles that were not thought to be dermal toxicants and Ag was chosen to provide an intermediate-effect material, whereby there have been no notifications of possible dermal irritation, however some literature suggests possible toxic effect in similar models (Carrola et al., 2016).

Methods

Test Materials and Preparation

The test materials used include ZnO NPs (NM110), TiO_2 NPs (NM105) and SiO_2 NPs (NM203) (JRC Nanomaterials Repository), CuO NPs (CuO NPs) (PlasmaChem GmbH, Germany), Ag NPs in 10 wt. % suspension (NM300K) (agpure W10, HeiQ RAS AG, Germany) and Ag nanopowder (Ag NPs, <100 nm particle size, contains PVP as dispersant) (Sigma-Aldrich product #576832, UK), which have all been previously characterised (Seleci et al., 2022, Singh et al., 2011, Rasmussen et al., 2014, PATROLS, 2018, Rasmussen et al., 2013, Centre et al., 2011, McKee et al., 2017). Testing of particle interference with assays is shown in supplementary information. Before use in HaCaT cell exposure assays, particles were suspended in sterile water at 5 mg/mL, briefly vortexed then sonicated for 10 minutes using a Ultrawave QS25 sonicating water bath operating at 400 J/sec. Stock solutions were vortexed for 20s then further diluted to 1 mg/mL in assay medium (medium composition detailed in subsequent section). For treatment of primary keratinocytes, test materials were prepared as a 5 mg/mL suspension, vortexed for 45 s and sonicated for 10 min using a vibracell 75,043 sonicator (Bioblock Scientific) equiped with a cup horn (Fisherbrand), and operating at 73% amplitude. In exposures of the RhE, particles were applied either as a powder, or as dispersions of either 400

mg/mL or 1 mg/mL. Dispersions were prepared at 400 mg/mL then vortexed for 20s before further diluting to 1 mg/ml in DPBS.

Table 1 Test material characterisation

Test Material	Formula Weight (g/mol)	Particle Size, TEM (nm)	BET Specific Surface Area (m^2/g)	Size distribution in HaCaT CCM: Z-Av (d.nm)	Size distribution in HaCaT CCM: PdI
ZnO	81.38	147 ± 149	12.4 ± 0.6	460.03	0.35
TiO_2	79.866	22.6 ± 1.4	46.175	357.70	0.36
SiO_2	60.08	68.64 ± 60.27	203.92	556.43	0.79
CuO NPs	79.545	24	34	191.07	0.41
Ag NPs	107.87	30.0 ± 23.9	6.43	445.97	0.53
NM300K	107.87	7.2 ± 4.3	38.1*	87.08	0.33

*calculated, not measured (McKee et al., 2017)

Study design

We have modelled our exposures of HaCaT cells and primary keratinocytes to complement the OECD TG 439 for Skin Irritation (OECD, 2020) exposure strategy, in which a short exposure time (1 hr) is used alongside a longer post-treatment incubation period; we added longer exposure times for HaCaT cells and primary keratinocytes to establish appropriate times for improved sensitivity, as required for robust SbD strategies. The assays were compared by a reduction in cell viability (as according to OECD TG 439 (OECD, 2020)), but also for the leaching of lactate dehydrogenase (LDH) to indicate cytotoxicity, and release of interleukin-1 alpha (IL-1α) to indicate pro-inflammatory responses. These two endpoints have long been considered relevant to human skin irritation *in vivo* (Perkins et al., 1999), and as such have often been used as biomarkers in skin irritation models (Gibbs, 2009). Moreover, they have been used to assess the impact of NMs on dermal models (Connolly et al., 2019, Chen et al., 2019), and IL-1α has been suggested by the ECVAM Scientific Advisory Committee (ESAC) to complement the MTT assay, possibly increasing sensitivity of the analysis and adding confidence in findings when a negative effect has been observed with use of the viability test (ISO, 2013). In addition, the HaCaT cell model was further used to understand the behaviour of our test materials in the scope of skin sensitisation, for which the model previously proposed and validated by Chung et al. (2018) and Jeon et al. (2019) was used. This approach allows for calculation of a stimulation index (SI) by measuring secretion of IL-1α and/or IL-6 from HaCaT cells following chemical exposure at precisely defined doses relating to dilutions starting from a 75% viable cell population; when an SI (calculated by cytokine produced in chemical-treated cells/cytokine produced in vehicle control) of ≥3 was achieved in multiple doses in either IL-1α or IL-6 secretion, the substance is predicted to be a skin sensitiser.

HaCaT cells

HaCaT cells (CLS cell lines service, Eppelheim, Germany) were maintained in Gibco™ Minimum Essential Medium (MEM) with GlutaMAX™ supplemented with 10% heat-inactivated Foetal Calf Serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin (all Thermo Fisher Scientific, UK) at 37°C with 5% CO_2 under sterile conditions. Cells were passaged twice a week.

Primary keratinocytes

Human primary keratinocytes (HPK) were prepared from human breast skin originating from plastic surgery. They were provided by Grenoble university hospital from healthy females with their

informed consent. For this study, young Caucasian type donors were selected (15-30 years old), bearing a phototype I or II according to the Fitzpatrick classification. After their isolation, HPK were grown in keratinocyte serum-free medium (KSF-M), to which was added 1.5 ng/mL of epidermal growth factor, 25 µg/mL of bovine pituitary extract and 75 µg/mL of primocin. They were maintained at 37°C, 5% CO_2 under sterile conditions. They were passaged once or twice a week depending on their density, and used at passage 1 or 2, then discarded as they acquired final differentiation and did not proliferate anymore. For cytotoxicity and ELISA assays, they were seeded in transparent 96-well plates at a density of 2.5 x 10^5 cells per well, then exposed to test materials in submerged condition 24 hr later. The full particle panel was not used in exposure of primary keratinocytes; for these comparisons we selected only particles which had previously demonstrated a strong positive response in HaCaT cells, and excluded particles which were shown to cause significant interference in assay outputs. The full panel tested in primary keratinocytes included Ag, CuO, ZnO and SiO_2 NPs.

Cell exposures in 96-well plates and viability assessment using alamarBlue

For submerged cell exposure experiments in 96-well plates (Sterile, flat bottomed; Thermo Fisher Scientific, UK), HaCaT cells were seeded at 3.5 x 10^5 cells/mL in 96-well plates (10.9 x 10^4 cells/cm^2). Plates were then incubated at 37°C, 5% CO_2, for 48 hr. HaCaT cells were exposed to test chemicals at concentrations of 7.8 – 500 µg/ml for assessment of cell death, and 7.8 – 125 µg/ml for pro-inflammatory mediator release. Negative controls contained medium only, and positive controls contained Sodium Dodecyl Sulfate (SDS) solution (0.02%, diluted from 10% solution in assay medium; Thermo Fisher Scientific, UK). The dosing strategy is shown in Figure 1; exposure time was either 1, 4, or 24 hr (4 hr data not shown). For 1 hr exposure time, after cells have been exposed medium was removed, wells washed once with medium and fresh medium added before returning to the incubator. After 24 hrs (total experiment time), supernatant was removed for subsequent analysis (LDH or cytokine analysis). Wells are washed twice with DPBS (Gibco™, Thermo Fisher Scientific, UK) then alamarBlue reagent was added (1.25% (v/v) solution of alamarBlue in serum-free, phenol red-free MEM (both Thermo Fisher Scientific, UK) prepared 24 hr before use and protected from light) and incubated for 60 minutes, protected from light. Fluorescence intensity was read at excitation/emission wavelengths of 532/590 nm for resorufin (Tecan Spark 10M plate reader for HaCat and SpectraMax ID3 from Molecular Device for HPK). AlamarBlue reagent was removed from the wells, before washing once with DPBS and adding fresh medium. Plates were incubated again for 23 ± 2 hr, when the alamarBlue assay was repeated. HPK were exposed using the same procedure except that only alamarBlue assay was applied. Although OECD TG 439 for Skin Irritation Test (OECD, 2020) suggests use of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay to determine cell viability, we have used alamarBlue throughout. MTT is not compatible with all NMs, and a number of NMs have been shown to interfere with the detection of cell viability when measured by MTT, including but not limited to Ag, carbon nanotubes and carbon black (Mello et al., 2020, Wörle-Knirsch et al., 2006, Kroll et al., 2012). Similarly to MTT, the alamarBlue assay provides a measurement of mitochondrial activity, and has been demonstrated as more sensitive than MTT in high throughput screening of chemical hazards (Hamid et al., 2004). For these reasons the alamarBlue assay was considered a better option for high throughput screening of NMs.

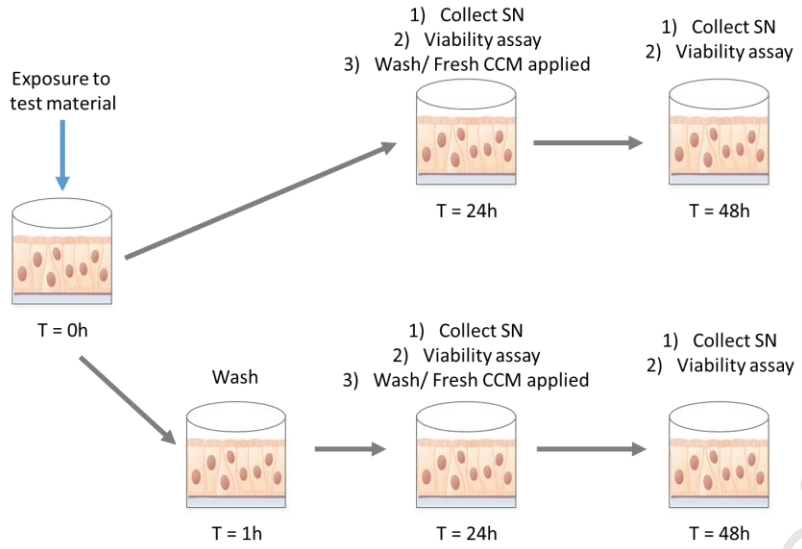

Figure 1 Dosing strategy for HaCaT cells and primary keratinocytes.

Cytotoxicity – lactate dehydrogenase (LDH) quantification

LDH activity was assessed by quantifying the conversion of a tetrazolium salt (iodonitrotetrazolium violet) into a red formazan product by released LDH in the supernatants collected from each exposure model. This was done using a commercially available kit (Cytotox 96® Non-Radioactive Cytotoxicity Assay, Promega), following the instructions provided. Absorbance was read at 490nm using a Tecan Spark 10M plate reader.

Pro-inflammatory response – determining cytokine release using Enzyme-linked immunosorbent assay (ELISA)

ELISA kits (Human IL-1α DuoSet, R&D Systems for HaCat cells and IL-1α Human ELISA kit, # BMS243-2TEN, ThermoFisher for HPK) were used to quantify the IL-1α cytokine concentrations following exposure to particles and control samples described above. Manufacturer's guidelines were followed using supernatants collected as described in the relevant particle exposure sections above. For HaCat, concentrations were determined by reading the absorbance at 450 nm (with reference wavelength of 550 nm) using a Tecan Spark 10M plate reader. For HPK, the absorbance at 450 nm (with reference wavelength of 650 nm) was measured using a SpectraMax ID3 (Molecular Device) plate reader. Human IL-6 ELISA kits (Invitrogen) were used to quantify the IL-6 cytokine concentrations following exposure of HaCaT cells to particle concentrations relevant to inducing 75% cell viability (CV75), 0.5 x CV75, 0.1 x CV75 and 0.01 x CV75.

OECD TG 439 Skin Irritation Test

Skin irritation was assessed using a 3D Reconstructed Human Epidermis (RhE) skin model (EpiDerm™, MatTek Life Sciences, Slovakia), following OECD TG 439 (OECD, 2020). Upon receipt of the RhE tissues, the inserts were visually inspected before moving to the top row of a new 6-well plate containing 0.9 mL fresh assay medium (EPI-100-ASY, MatTek Life Sciences, Slovakia). Plates were then incubated for a 60 ± 5 min at 37 ± 1° C, 5 ± 1% CO_2, relative humidity (RH). After this time, inserts were transferred from the upper wells to the lower wells of the 6-well plate containing fresh assay medium. Plates were placed back into the incubator (37 ± 1° C, 5 ± 1% CO_2) for an overnight pre-incubation (25 ± 1 hr).

After moistening the tissues with 25 µl DPBS (MatTek Life Sciences, Slovakia), tissues were treated with test chemicals as received as dry powders (using a spoon; NaCl weight: 25 mg) or as liquid dispersions (30 µL) and exposed for 1 hr. Negative control was DPBS only and positive control was 5% SDS (10% solution diluted with sterile H_2O), both applied at a volume of 30 µL. After 1 hr, tissues were thoroughly washed with DPBS (Gibco™, Thermo Fisher Scientific, UK), blotted to remove any remaining liquid, transferred to a new 6-well plate pre-filled with 0.9 mL assay medium and incubated for 24 ± 2 hr (37 ± 1° C, 5 ± 1% CO2, 95 % RH). After 24 ± 2 hr post-incubation, inserts are transferred into the lower part of the 6-well plate, pre-filled with 0.9 ml of media. Media from upper rows were collected for analysis of the additional endpoints (LDH and IL-1α secretion, not OECD guidance compliant). Plates were returned to the incubator (37 ± 1° C, 5 ± 1% CO_2) for the final post-incubation (18 ± 3 hr).

After the final post-incubation was complete, 300 µL of alamarBlue solution (1.25% (v/v) solution prepared as described previously) was added into each well of a 24-well plate. Tissues were removed from the incubator, blotted to remove any assay medium, then transferred to the 24-well plate pre-filled with alamarBlue solution. The plate was then added to the incubator (37 ± 1° C, 5 ± 1% CO_2) for 3 hr ± 5 min. After this time, two aliquots (100 µL) were removed for each tissue and transferred to a 96-well plate, then the fluorescence intensity was read at excitation/emission wavelengths of 532/590 nm for resorufin (Tecan Spark 10M plate reader).

Test substances are classified as dermal irritants if the mean tissue viability was lower than 50%. This was determined by calculating the relative viability of test substances according to their results in the alamarBlue assay:

$$Relative\ viability\ (\%) = \frac{Fluorescence\ intensity\ of\ test\ substance}{Average\ fluorescence\ intensity\ of\ negative\ control} \times 100 \qquad (1)$$

Dose application relevant to Skin Irritation Test

The OECD TG suggests application of a substance should be in the form that it is received (i.e. as a powder if received as a powder). However, when investigating the available information on dermal irritation of NMs within literature, we noted that the vast majority of studies applied NMs in a dispersion prepared up to 1 mg/mL, and that many of these exposures resulted in no effect being observed. We wanted to test what impact this divergence from the test guideline would have on our benchmark materials. Following the OECD TG 439, 30 µL of a suspension should be added; 30 µL of a 1 mg/mL dispersion applied is approximately only 30 µg, which is far less than the amount that is recommended when applying a dry powder, which should be applied using a spoon calibrated to 25 mg of NaCl. We investigated the effect this has on the irritation potential of CuO, as we had already found this test particle fulfilled the requirements for irritant classification as an applied powder. To do this, we applied CuO as a dry powder, and in a dispersion using 30 µL of a high concentration (400 mg/mL) and a low concentration (1 mg/mL) in DPBS; it was not possible to attain a dispersed dose similar to the powder dosing, therefore we used the highest practicable dose.

Statistical Analysis

All data are displayed as mean averages ± standard error of mean (SEM), with at least 3 biological replicates. Statistical analysis was performed using IBM's SPSS Statistics (version: 29.0.0.0 (241)). Normal distribution of data was assessed using the Kolmogorov-Smirnov normality test and Normal Q-Q Plot. Data considered to have normal distribution were analysed using Ordinary one-way ANOVA with Tukey post-hoc for multiple comparisons. Data not considered to have normal

distribution were instead analysed using Kruskal-Wallis test with pairwise comparisons. Values of $p \leq 0.05$ were considered statistically significant.

CV75 (i.e. the test chemical concentration that results in 75% cell viability (CV)) was determined using the method outlined in OECD TG 442E (OECD, 2022), by first calculating the relative viability at each test concentration applied following Equation 1, then determining the CV75 value by log-linear interpolation using the following equation:

$$Log\ CV75 = \frac{(75-c) \times Log\ (b) - (75-a) \times Log\ (d)}{a-c} \quad (2)$$

Where *a* is the minimum value of cell viability over 75%, *c* is the maximum value of cell viability below 75%, and *b* and *d* are the concentrations showing the value of cell viability *a* and *c* respectively.

Results

Viability

ZnO, CuO and NM300K particles showed significant reduction in HaCaT cell viability following 24 hr exposure (Figure 2), with <50% viability observed at particle concentrations of as low as 31.25, 62.5 and 62.5 µg/ml, respectively. For the Ag NPs, a steady decline in cell viability was observed with cell death reaching 50% at approximately 250 µg/ml. SiO_2 resulted in a slight reduction in cell viability at concentrations of 62.5 µg/mL or higher, however cell death only reached a maximum of 25% at the highest concentrations. TiO_2 showed no effect on cell viability after 24 hr exposure. At all exposure times, the positive control (SDS at 0.02%) was shown to fulfil the criteria for an irritant as set out in OECD TG 439 (<50% viability).

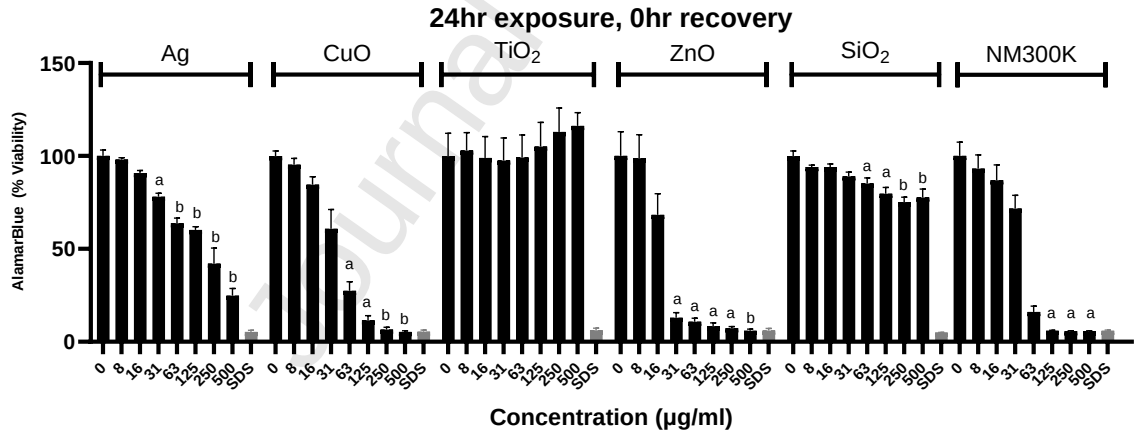

Figure 2 Viability of HaCaT cells following 24 hr exposure to Ag NPs, CuO NPs, TiO_2, ZnO, SiO_2 and Ag NM300K with no recovery time. A range of particle concentrations were tested (7.81-500 µg/ml). Positive control (grey bars) is 0.02% SDS. Comparison to medium only control: a = p ≤ 0.05, b = p ≤ 0.001.

A similar effect was observed for the 1 hr exposure time for ZnO, CuO and NM300K; however, for Ag NPs 50% cell death was only achieved at the highest concentration for 1 hr exposure time. This pattern was reproduced when a 23 hr recovery time was included in the protocol (Figure S1). Based on the results presented in Figure 2 and Table 2, a ranking of the nanoparticles in terms of effect on HaCaT cell viability would be TiO_2 (least effect on cell viability) < SiO_2 < Ag NPs < CuO ≈ NM300K < ZnO (greatest effect on cell viability). When dosimetry is converted to m^2/L using the BET surface area of the particle, a different ranking is achieved: TiO_2 (least effect on cell viability) < SiO_2 < CuO ≈ NM300K < Ag NPs < ZnO (greatest effect on cell viability) (Figure S2).

Table 2 75% cell viability (CV75) values for viability of HaCaT cells exposed to Ag NPs, CuO NPs, TiO_2, ZnO, SiO_2 and Ag NM300K for 24 hr with no recovery time.

CV75	Ag NPs	CuO	TiO_2	ZnO	SiO_2	NM300K
µg/mL	56.57	24.66	>500	9.713	146.36	20.03
m^2/L	0.364	0.838	>23.088	0.120	29.845	0.763

In comparison to the HaCaT stable cell line model, we tested a smaller particle panel in primary keratinocytes, and selected particles which had previously produced a strong positive response and were not shown to elicit high levels of interference in assay outputs; primary keratinocytes were exposed to Ag, CuO, ZnO and SiO_2 NPs using the same treatment regime and concentration range as was used for HaCaT exposures (Figure 3 and Figure S3). There was no effect evident at 1 hr in any treatment. ZnO NP were shown to reduce cell viability considerably at all other time points, SiO_2 demonstrated a dose-dependent cell death which increased over the exposure time points, CuO was only found to reduce cell viability at highest doses, and Ag NP also induced a dose-dependent cell death that increased over the time points.

Interference with detection of responses by the alamarBlue assay is shown in Figure S4; where only NM300K was shown to impede this measurement, and only at the highest concentrations.

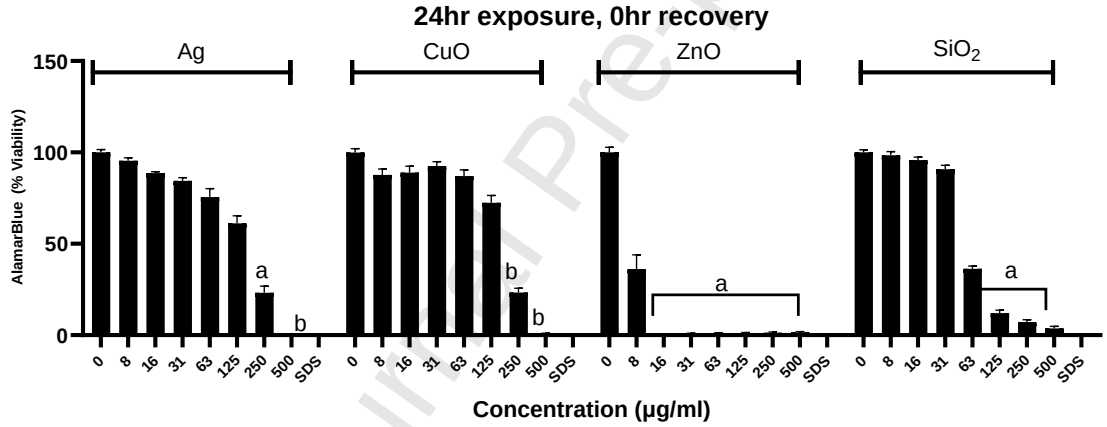

Figure 3 Viability of primary keratinocytes cells following 24 hr exposure to Ag NPs, CuO NPs, ZnO, and SiO_2. A range of particle concentrations were tested (7.81-500 µg/ml). Positive control (grey bars) is 0.02% SDS. Comparison to medium only control: a = $p \leq 0.05$, b = $p \leq 0.001$.

Cytotoxicity

LDH secretion by HaCaT cells for a 1 hr exposure is shown in Figure 4, and 1 hr with recovery period and 24 hr exposures in Figure S5; significant responses were observed for ZnO, CuO, TiO_2, Ag NPs and NM300K after 1 hr exposure. This LDH release was shown to reduce after 23 hr recovery for CuO, ZnO and NM300K, this is likely to be due to high levels of cell death observed at the early time point, or for CuO and NM300K this may be due to particle interference (supplementary information). Despite not observing high levels of cell death in the viability assay, significant responses are observed following exposure to TiO_2, both with and without recovery, and SiO_2 induced significant release with 1 hr exposure and recovery of 23 hr. Results from the cytotoxicity assessment of HaCaT cells exposed to the positive control (0.02% SDS) did not show a significant effect after 1 hr exposure, despite the viability results showing a high concentration of cell death.

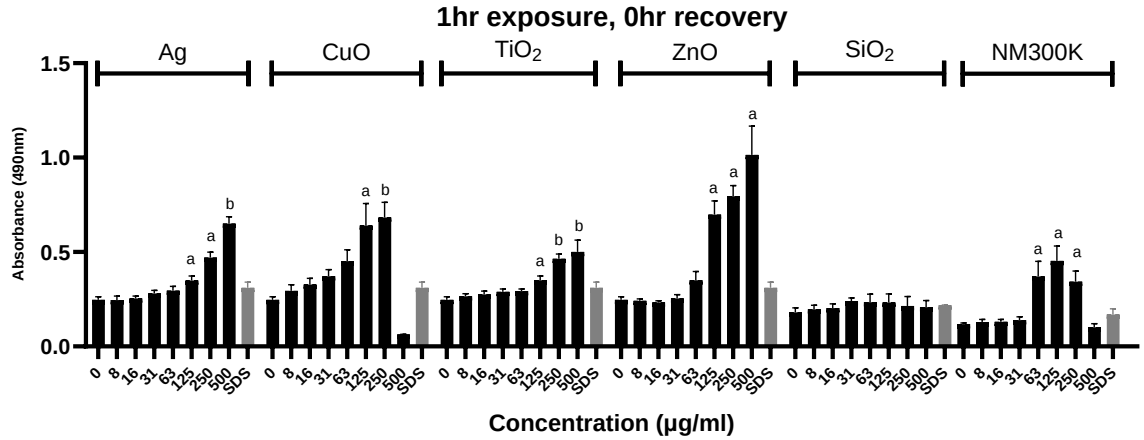

Figure 4 Cytotoxicity of HaCaT cells, measured by LDH release, after exposing to Ag NPs, CuO NPs, TiO_2, ZnO, SiO_2 and Ag NM300K particles at a range of concentrations (8 – 500 ug/ml) for 1 hr, without a recovery time. Positive control (grey bars) is 0.02% SDS. Comparison to medium only control: a = p ≤ 0.05, b = p ≤ 0.001.

As discussed for the viability results, the dosimetry units used appears to have an effect on the ranking of test chemicals with respect to increase LDH release (Figure S6). This effect was most apparent when looking at the results for SiO_2, TiO_2 and Ag NPs after 23 hr recovery, whereby ranking using mass-based dosimetry would assign all particle equal toxic effects, whereas when ranked using dosimetry based on surface area of the particle, Ag NPs show greatest effect followed by TiO_2 then SiO_2.

Interference of the particle panel with the LDH assay is shown in Figure S7. NM300K was shown to interfere considerably in optical detection of assay readouts, while CuO NPs appeared to deplete detectible level of LDH presence; this was observed only when incubated with LDH for 24 hr, not observed when incubated together for 4 hr.

Pro-inflammatory Response

The release of the pro-inflammatory mediator IL-1α was assessed in both the HaCaT cell line and in primary keratinocytes. Release of IL-1α from HaCaT cells was observed in response to SiO_2, ZnO, Ag NPs, NM300K and CuO; in general this was observed at each exposure time and throughout the 23 hr recovery time, which resulted in an exacerbated response (Figure 5 and Figure S8). Considerable interference was observed when using NM300K in these assays (Figure S11), as such these results for NM300K may be an underestimation. In the exposure of primary keratinocytes (Figure 6 and Figure S9), there was minimal IL-1α secretion at 1 hr, which increased in response to ZnO, and SiO_2 for all subsequent time points, and to CuO at only the longest exposure (24 hr plus 23 hr recovery). Ag NPs did not induce any cytokine release of note. The considerable cell death observed for SiO_2 and especially ZnO would have greatly affected the cytokine release here, as cell numbers would have been low.

In addition to this IL-1α secretion, we also studied the release of IL-6. However, this was done under a different context, and related to the proposed use of HaCaT cells as a tool to identify skin sensitising agents (Jeon et al., 2019, Chung et al., 2018). The previously described experiments determining cell viability of HaCaT cells were used to calculate CV75 values of each particle exposure (Table 2). With dilutions of Ag NPs, CuO, ZnO and SiO_2 to x0.5, x0.1 and 0.01 of the dose required for CV75, new exposures were performed and supernatant analysed for secretion of IL-6. After 24 hr exposure SiO_2 induced statistically significant release of IL-6 at 1 x CV75 and 0.5 x CV75, while no other material caused a significant change (Figure 7). When a 23 hr recovery was included in the

protocol release of IL-6 was observed with SiO_2 at 1 x CV75 and 0.5 x CV75, Ag NPs at 1 x CV75, and CuO at 1 x CV75 all after 24 hr exposure to cells. In each of these cases, an SI index of ≥3 was also observed (Figure S10).

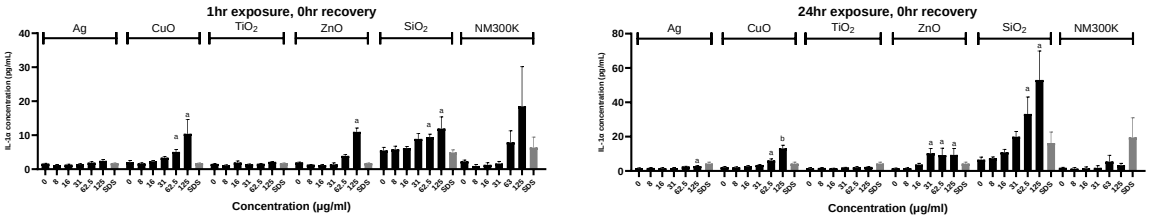

Figure 5 Release of IL-1α from HaCaT cells after exposing to Ag NPs, CuO NPs, TiO_2 NM-105, ZnO NM-110, SiO_2 NM-203 and Ag NM300K particles at a range of concentrations (8 – 125 ug/ml) for 1 hr and 24 hr, each with either no recovery time recovery time. Positive control is 0.005% SDS. Comparison to medium only control: a = p ≤ 0.05, b = p ≤ 0.001.

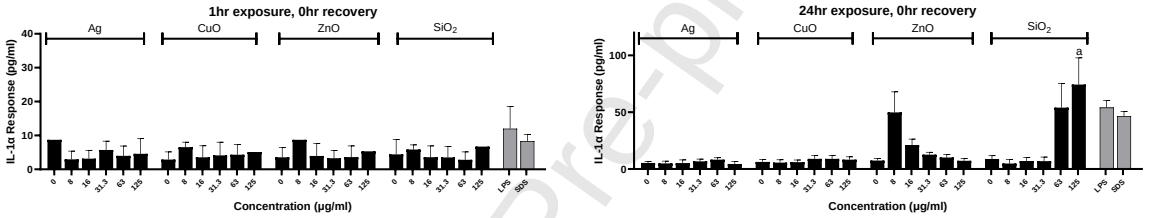

Figure 6 Release of IL-1α from primary keratinocytes after exposing to Ag NPs, CuO NPs, ZnO NM-110, and SiO_2 NM-203 particles at a range of concentrations (8 – 125 ug/ml) for 1 and 24 hr exposure. Positive control is 0.005% SDS and LPS. Comparison to medium only control: a = p ≤ 0.05.

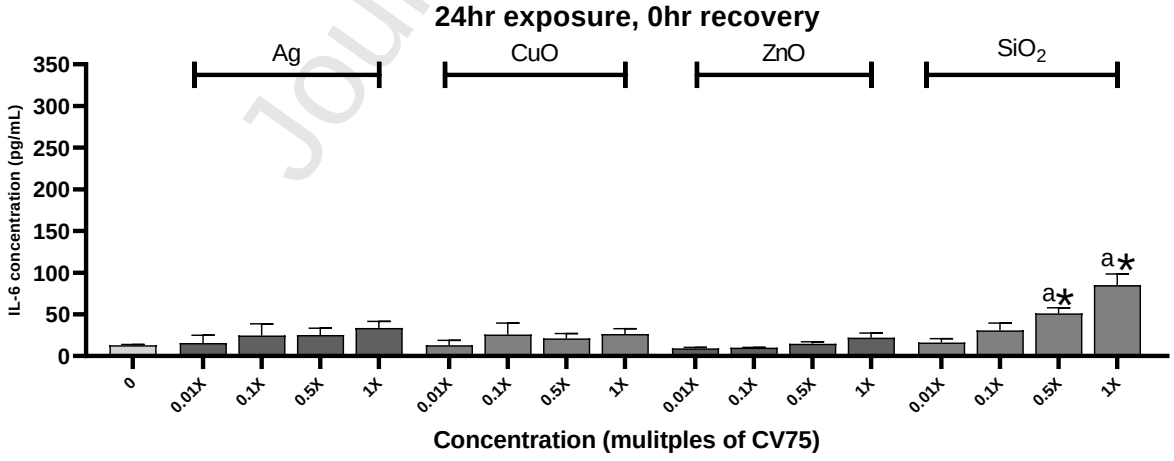

*Figure 7 Release of IL-6 from HaCaT cells after exposing to Ag NPs, CuO NPs, ZnO NM-110, and SiO_2 NM-203 particles at a range of concentrations derived from previously determined CV75 values, including 0.01 x CV75, 0.1 x CV75, 0.5 x CV75, and 1 x CV75 for 24 hr. CV75 (in µg/mL) for Ag = 56.57, CuO = 24.66, ZnO = 9.71, and SiO_2 = 146.36. Statistical differences compared to medium only control: a = p ≤ 0.05; identification of when treatment had a SI index of ≥3 shown with *.*

Skin Irritation and RhE toxicity

When testing the benchmark materials and controls following the OECD TG 439 irritation method, the positive control (5% SDS), CuO NPs, and NM300K all induced statistically significant reduction in cell viability, albeit only the positive control and tissues exposed to CuO NPs met the criteria to be classified as an irritant Figure 8. In the assessment of other endpoints relating to general toxicity, significant LDH secretion from RhE tissues was observed in response to NM300K after 1 hr, this diminished and was not evident at later time points. There were no significant responses compared to the negative control at 24 hr. However, after 42 hr a significant increase in LDH secretion for tissues exposed to CuO was observed (Figure 8). An increase in IL-1α release was observed for tissues exposed to the positive control at 24 hr, this was no longer evident after 42 hr. Whereas, after 42 hr a significant increase in IL-1α was observed in tissues exposed to CuO NPs (Figure 9).

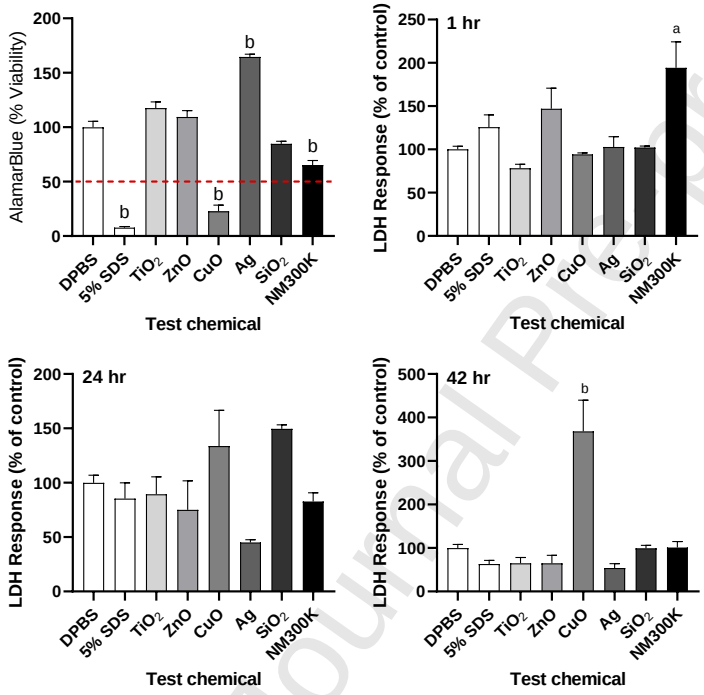

Figure 8 Cell viability and LDH release from of RhE tissues after exposure to benchmark particles TiO_2 NM-105, ZnO NM-110, CuO NPs, Ag NPs, SiO_2 NM-203 and Ag NM300K for 1 hr, with post-treatment recovery times of 42 hr for alamarBlue, and 1, 24, and 42 hr for LDH release. Controls included 5% SDS (positive control) and DPBS (negative control). Particles deposited onto tissues as received. Comparison to negative control: a = $p \leq 0.05$, b = $p \leq 0.001$.

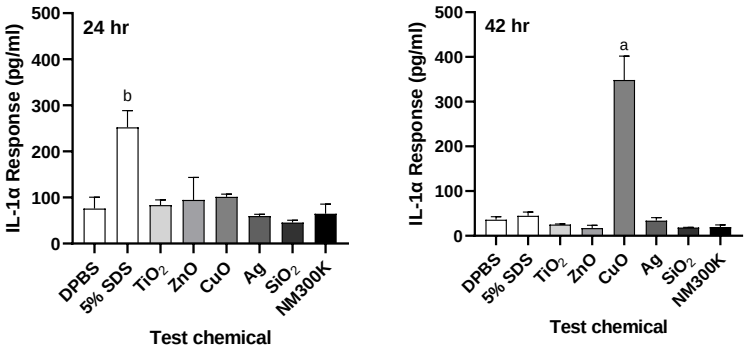

Figure 9 Release of IL-1α from RhE tissues after exposing to benchmark particles TiO_2 NM-105, ZnO NM-110, CuO NPs, Ag NPs, SiO_2 NM-203 and Ag NM300K for 1 hr. Controls included 5% SDS (positive control) and DPBS (negative control). Measurement taken from supernatant collected 24 and 42 hr after exposure. Particles deposited onto tissues as received. Comparison to negative control: a = p ≤ 0.05, b = p ≤ 0.001.

The comparison of different sample application in respect to the skin irritation protocol is shown in Figure 10. When following more precisely the OECD irritation protocol, using both the dry powder and the dispersion at high concentration resulted in >50% reduction in cell viability, i.e. CuO is classed as a dermal irritant, whereas, when tested at the low concentration, CuO no longer reached the requirements for classification. The measurements of LDH release and IL-1α secretion followed this same pattern, the dry powder application and that of the dispersion at high concentration resulted in comparable results.

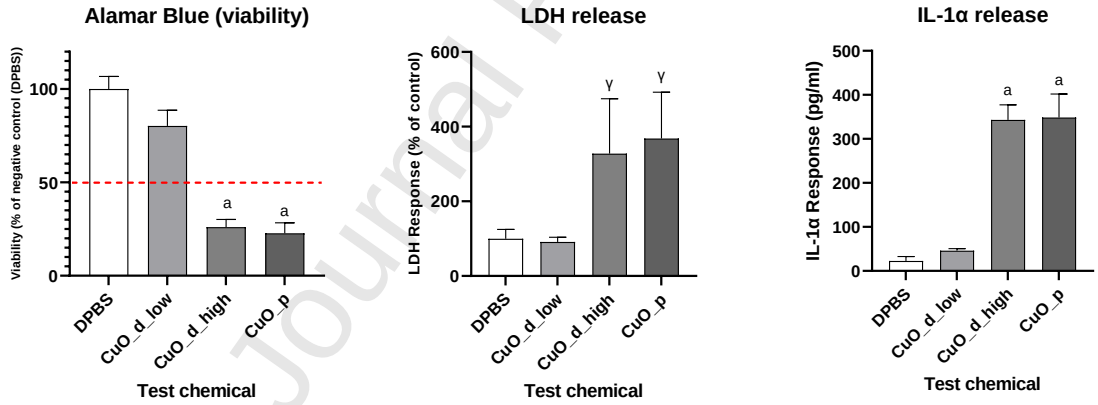

Figure 10 Dermal irritation to RhE tissues after exposure to CuO as a dry powder (CuO_p),30 µL of 400 mg/mL dispersion in DPBS (CuO_d_high) or 30 µL of 1 mg/mL dispersion in DPBS (CuO_d_low). Negative control is DPBS. Tissue viability measurement 42 hr after exposure, LDH and release of IL-1α used supernatant collected 42 hr after exposure. Statistical significance identified in comparison to negative control: a = p ≤ 0.05, and in comparison to 'low' concentration CuO dispersion: γ = p ≤ 0.05.

Discussion

In this study, a number of benchmark NMs were used to predict hazards of dermal exposure to NMs and to compare different cellular models, determining which may be appropriate and useful for SbD decisions during stages of early-innovation. The benchmark substances were TiO_2 NM-105, ZnO NM-110, CuO NPs, Ag NPs, SiO_2 NM-203 and Ag NM300K. These benchmark NMs were used to generate a varying range in toxicity that could be used to correlate responses between a stable cell line (HaCaT cells), primary keratinocytes, and RhE; given the relative simplicity, and the practical time-

and cost-saving in using the HaCaT model, it was proposed as the desired model for use in early-stage innovation SbD decision-making. In addition, we performed a dosing strategy comparison to better understand discrepancies within the literature in performing testing of NM effects on RhE in relation to OECD test guidance. The treatment regime mostly used throughout this study was based on the OECD test guidance for skin sensitisation (OECD, 2020), which employs a treatment time of up to 1 hr followed by a recovery period of up to 42 hr; for cell line exposures we extended the treatment time to also include a 24 hr exposure.

Consideration of the conditions used for the HaCaT cell model as a tool for SbD decision-making. The 1 and 24 hr exposure, each without recovery time, were found most appropriate when assessing cell viability. These times allowed an identification of substances showing a particularly rapid response in 1 hr (i.e. both Ag NPs, CuO, and ZnO), the development of a dose-response within 24 hr for each material where an effect was noted, and identification of more slowly developing responses over 24 hr. The delayed response of some (e.g. SiO_2 NPs) could be due to either the response time for these particles to induce this effect being greater than 1 hr, or particle deposition onto the cell surface is delayed. Hence the 24 hr exposure time allowed sufficient deposition and/or time for effects on cell viability to occur. The inclusion of the 4 hr exposure and the 23 hr recovery time were not beneficial in the cell death assessment of HaCaT cells as no significant change in effect was observed for any of the test particles. The use of 0.02% SDS as a positive control for this assay was shown to be effective, resulting in almost complete cell death. In general, these data largely correspond with results reported in literature, where exposure of HaCaT cells to CuO and ZnO for 24 hr was shown to induce high levels of cell death at concentrations >30 µg/mL (Alarifi et al., 2013, Jang et al., 2012, Vinardell et al., 2017). A study on Ag NPs toxicity to HaCaT cells found reduction in cell viability after 24 hr exposure that varied depending on the surface coating of the Ag NPs, where 40 µg/mL resulted in >50% reduction in cell viability in some instances (30 nm Ag NPs with citrate surface layer, with and without a coating of bovine serum albumin) but in others cell viability was approximately 65 – 80 % (Carrola et al., 2016). The authors note that the mode of toxicity for Ag involves uptake of Ag NPs to the cells followed by intracellular release of Ag+, whereas Ag+ formed outside of the cell is not as readily taken up by cells so the mechanism of toxicity is largely due to extracellular processes resulting in cell membrane damage. Therefore, the rate of uptake and rate of dissolution will likely be driving factors for Ag NP cytotoxicity and could be responsible for the minor differences in results for Ag NPs and NM300K used in the current study. Similarly, Kim et al. (2021a) discuss the cytotoxic results they observed for CuO and ZnO to KeratinoSens™ cells could be due to the ions released *via* dissolution of the respective nanoparticles. The ion concentration after 24 hr in assay medium was not determined in our assessment so we are unable to comment on how this may affect the results obtained. In comparison of the two different Ag NPs used in our study, it appeared that NM300K induced greater cell death, however, this was hindered by the interference of NM300K in the viability assay, especially at high concentrations. Cytotoxicity assessment by LDH secretion showed further interesting results with respect to varying exposure and recovery times. It would appear that 1 hr exposure is sufficient to induce a significant response for LDH secretion in all test chemicals, including TiO_2, which had not shown an effect in the WST-1 assay. Increasing the exposure time from 1 hr and 24 hr did result in an enhanced effect for ZnO and SDS (0.02%); however, for particles where an interference was noted with assay reagents (namely CuO and NM300K) results actually appeared to be negatively affected and a reduction in LDH secretion compared to 1 hr exposures was noted. This is likely a result of the inclusion of an additional washing step in the 1 hr exposure time protocol, hence reducing the interference effect. Inclusion of the 23 hr recovery time allowed a dose-response effect to be noted for SiO_2, which was not yet observed without the recovery time. However, for particles such as CuO, NM300K and ZnO where cell viability was greatest, there was no

benefit to including the results with the 23 hr recovery time as significant levels of cell death resulted in a reduction of LDH secretion compared to results without recovery time. Using 0.02% SDS as the positive control does not appear suitable for this assay, as results from the 1 hr exposure did not result in elevated responses with respect to the negative control. Therefore, selection of one of the test materials used in this study may be more suitable. Increasing the exposure time from 1 hr to 24 hr provided no additional value for test substances, such that all responses observed at 24 hrs were already apparent after 1 hr exposure. Looking further into the comparison between 0 hr and 23 hr of recovery time after exposure, we note that the inclusion of a recovery time is not appropriate for LDH analysis if there has been significant cell death (e.g. ZnO, CuO, NM300K and SDS). However, there is value in including the recovery time for particles such as SiO_2, TiO_2 and Ag, whereby the toxic effects appear to occur over a longer timeframe. This may be a reflection of the mode of toxicity, *i.e. via* ions or by particles, however more investigation would be required to determine this. With IL-1α, as with other IL-1 proteins, being constitutively expressed by cells (Cavalli et al., 2021) a rapid secretion may be expected, which was demonstrated here with secretion detected in response to numerous benchmark substances after just 1 hr exposure. However, the selectivity and sensitivity was increased through either increasing exposure time or recovery time. Therefore, it demonstrates the importance of all conditions to reflect the diversity in responses possible. Due to the rapid cell death induced by 0.02% SDS it was deemed inappropriate as control in these assays, even at sub-lethal concentrations of 0.0005% there was still no clear IL-1α secretion, and again a more suitable control would be to select one of the test particles when implementing this protocol in future.

We further assessed the HaCaT cell model under the conditions proposed for *in vitro* determination of skin sensitising agents, based on work published by Jeon et al. (2019) and Chung et al. (2018). According to this method, treatments are applied to HaCaT cells within a strict dosing strategy, with a highest dose causing CV75 and dilutions from this to achieve 0.5 x CV75, 0.1 x CV75, and 0.01 x CV75. After 24 hr exposure it is determined whether any of these treatments have caused an SI ≥ 3 in respect to IL-1α or IL-6 release, i.e. whether treatments induced a response three times that of untreated cells. If this is shown through either cytokine, the material is classified as a sensitizer, if shown to neither, it is not. Although a number of our previous exposures had shown significant IL-1α release, e.g. CuO, Ag, ZnO and SiO_2, it was clear that under the revised dose regime there would no longer be a response to CuO, Ag, and ZnO, given the CV75 value threshold. Therefore, we looked no further into IL-1α and focussed on IL-6. Under these conditions, only SiO_2 was shown to induce IL-6 to three times that of background controls, and would therefore be classified as a sensitising agent, which is interesting given that it has been proposed that sensitization is likely to be related to the surface reactivity of a particle (Di Cristo et al., 2022), and the SiO_2 used, being fumed silica, is a particularly reactive particle. The other NMs tested (Ag, CuO and ZnO) had shown no such response. These data did not fully align with more dedicated sensitisation assays used elsewhere. Ag and ZnO NPs were both shown as skin sensitizers in the h-CLAT assay by Gautam et al. (2021), when we did not, Ag NPs were also shown as a weak skin sensitiser according to OECD TG 406 by Kim et al. (2013). Again in contrast to the data presented here, CuO NPs were shown as skin sensitizers in the ARE-Nrf2 Luciferase KeratinoSens™ assay, while similarly to the data here, ZnO NPs were not (Kim et al., 2021a). SiO_2 was shown by Kim et al. (2021b) to not cause skin sensitization, although this was when using colloidal silica, in our case we were exposing cells to fumed silica. It is not clear where these differences originate from, possibly through differences in particles used, or through the robust cytotoxicity of the test panel used here in HaCaT cells making analysis of cytokine release difficult.

Comparison of the HaCaT cell model to primary keratinocytes. Four benchmark materials were selected to compare the HaCaT cell model to primary keratinocytes, including Ag NPs, CuO, ZnO and

SiO_2; there were considerable differences in the manner in which HaCaT cells and primary keratinocytes responded to these NMs. HaCaT cells were far more rapidly susceptible to particles exposures, with reduced HaCaT cell viability observed within 1 hr in response to Ag NPs, CuO and ZnO, not observed in primary keratinocytes. This may be due to the quicker cell cycle of HaCat cells compared to primary keratinocytes, the metabolism is certainly much more active in HaCat than in human primary keratinocytes (their doubling time is rather 2 or 3 days than 24 h). Another explanation can also be the different cell culture medium, which may dissolve some of the particles more or less rapidly compared to HaCaT cell exposure medium. Over the next three exposure time points, the response of both cell types was shown to be similar for AgNPs, while for ZnO and SiO_2 NPs the primary keratinocytes had considerably greater cell death than HaCaT cells, and, conversely, CuO induced far less cell death in primary keratinocytes compared to HaCaT cells. In respect to their pro-inflammatory response, the significant IL-1α secretion by HaCaT cells in response to AgNPs at later time points was not shown for primary keratinocytes. CuO NPs also induced considerable IL-1α secretion in HaCaT cells which was also not observed in primary keratinocytes. It is possible that this may be due to the lack of cell death caused by CuO in primary keratinocytes, as one mechanism for IL-1α release is through cell necrosis (Cavalli et al., 2021). SiO_2 NPs induced IL-1α secretion in both cell types, albeit far more vigorously by HaCaT cells. Any pattern of IL-1α secretion by either cell type in response to ZnO NPs was difficult to interpret, as we believe it was too hampered by considerable and rapid (particularly for HaCaT cells) cell death.

Comparison of cell cultures to the RhE tissue. A key question in this study was whether either of the *in vitro* cell systems tested correlated to toxicity endpoints achieved using the OECD dermal irritation protocol with RhE tissues; an overview of model comparison can be seen in Table S1. The 2D cell models were found to be more sensitive to particle exposure than the RhE tissues, particularly for ZnO, Ag NPs and NM300K in HaCaT cells, and ZnO and SiO_2 in primary keratinocytes. However, cell viability reduction was found similar in RhE and HaCaT cells in respect to CuO NP exposures, which was identified as an irritant in both models; it is this material which may be suitable for use as a positive control in future work. Despite showing cytotoxic effects in the cell models, ZnO NPs did not result in any significant reduction in tissue viability, and Ag NPs showed an unexplained significant increase in RhE cell viability rather than causing any reduction, as was shown in HaCaT cells. SiO_2 and NM300K did show a reduction in viability compared to the negative control but not enough to be classified as irritants (85% and 65%, respectively). Our results align with those reported by Jang et al. (2012), whereby they observed cytotoxicity to HaCaT following exposure to ZnO (size- and dose-dependent effects) but not when applied to the same RhE model as we have tested (ZnO applied as a dispersion at 50 µg/mL). Similarly, Vinardell et al. (2017) also found cytotoxic effect to HaCaT from ZnO (concentrations up to 100 µg/mL) but no reduction in viability in an irritation test using a RhE model (24 h treatment with 500 µg/mL). Further similarities, and dissimilarities, between RhE tissue and HaCaT cells were shown in IL-1α in response. While SiO_2 induced high levels of IL-1α in HaCaT cells, none was found in RhE tissue expsoures. However, CuO NPs were shown to induce significant release over time in both HaCaT cells and RhE, again not replicated in use of primary keratinocytes. Consideration must be given to the concentrations of CuO chosen for the positive control to ensure that interference with assay reagents does not affect the results obtained, which was most considerable for the LDH assay. It is recognised that the application of particles to the RhE tissues is more realistic to human dermal exposure than the simple 2D models. However, it is worth noting that the simple 2D model is not expected to be used as a replacement for the dermal irritation protocol as this is already a regulatory accepted method, and mechanistically cannot be replicated in the simple 2D system given that dermal irritation is induced by a particle's ability to both penetrate the stratum corneum and to induce cytotoxicity (Gibbs, 2009). Instead, the HaCaT model should be

used to prioritise particles for further assessment. It is still valuable, however, that for some treatments, particularly CuO (proposed for use as a positive control), results have been well correlated between these two models.

In addition to the model comparisons, we also assessed the application of NMs to RhE models and note the importance of applied concentration to RhE tissues. In the OECD protocols it is recommended to apply test substances as received. However, for NMs this can prove difficult due to their phys-chem properties making handling problematic. Therefore, to improve ease of testing and reproducibility, it would be preferable to apply NMs in a dispersion. As noted, this is common in the studies reported in literature (Jang et al., 2012, Park et al., 2011, Miyani and Hughes, 2017). However, the concentration of particle dispersions applied seems to vary greatly and the maximum concentration noted in these studies was 1 mg/mL, although this is in line with typical *in vitro* cell exposures, it may not reflect the concentrations applied when following the OECD recommendation of dry powder use. In a comparison of three different application approaches, as dry powder (OECD recommended), as a 1 mg/mL, or as a high-concentration dispersion (400 mg/mL), we were able to replicate the RhE response to CuO NPs as a powder application using a similar dose applied as a high-concentration dispersion, while the lower dose of 1 mg/mL was found dissimilar. Therefore, we suggest that these higher dispersion concentrations should be used when NMs are applied in the RhE model.

Conclusion. It is clear that there is not full alignment with these models, but there still appears to be merits in using simpler models, with the HaCaT model being more closely aligned, than primary keratinocytes, to responses of RhE. Therefore, it is the HaCaT cell model which we would propose as a suitable model for screening of dermal toxicity of NMs and use for SbD decisions during early stages of innovation. Within the testing regime used for HaCaT cells there can be a number of recommendations for use. These include the use of necessary endpoints (viability, cytotoxicity and immune response) to allow control of possible interferences, the use of specific exposure times, including both the 1 and 24 hr exposures, both with and without the 23 hr recovery time, as these allowed for the investigation of materials with a rapidly- *versus* slowly-developing response, and that CuO NPs provide a robust positive control, with responses reproduced in cell line and RhE tissues.

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Conflicts of Interest
The authors declare no conflict of interest.

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights:

- The *in vitro* HaCaT cell model provides better support for hazard screening of nanomaterials than primary keratinocytes.
- Early-stage safe-by-design decisions can be made with data collected with the *in vitro* HaCaT cell model.
- When assessing dermal toxicity endpoints, the *in vitro* HaCaT cell model correlates more closely with the regulatory accepted reconstructed human epidermis (RhE) model than primary keratinocytes do.
- The dosing strategy used for nanomaterials can greatly affect the outcome when using the OECD TG for *in vitro* skin irritation.