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Experimental approaches to data generation for REACH compliance of multi-walled carbon nanotubes: human health *in vitro/in chemico*

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Applying regulatory-accepted, standardised test guidelines to solid, non-dispersible nanomaterials is challenging, primarily due to the difficulty of generating a homogenous exposure medium. Additional challenges arise from the physicochemical characteristics of multi-walled carbon nanotubes (MWCNTs), which are typically light and exist as entangled bundles. This study evaluated the applicability of available standardised *in vitro/in chemico* OECD test guidelines (TGs) for animal-free human health testing and explored potential adaptations to make them suitable for MWCNTs. Our focus was on EU-REACH data requirements related to *in vitro* serious eye damage/irritation, *in chemico* skin sensitisation, and *in vitro* gene mutation in mammalian cells. We assessed the applicability of OECD TG 492B, TG 442D/442E, and TG 476 for these endpoints. Our findings indicate that adequate data may only be generated if solid nanomaterials can be applied as such (as in OECD TG 492B), or if nano-specific dispersion protocols are available for an endpoint (as in genotoxicity testing), whereas significant limitations remain for skin sensitisation testing.

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Environmental significance

Generating safety data for carbon-based nanomaterials such as multi-walled carbon nanotubes (MWCNTs) is challenging due to the lack of standardised test guidelines for both human health and ecotoxicity endpoints. Our research focusses on fulfilling REACH data requirements by exploring the use of new approach methodologies (NAMs) to assess nanotoxicity. Specifically, we evaluated the applicability of newly accepted *in vitro/in chemico* test guidelines for eye irritation and skin sensitisation to MWCNTs. Our work aims to establish non-animal testing strategies that can fulfil regulatory data requirements, directly reducing the need for *in vivo* animal studies. This represents a step toward streamlining chemical risk assessment for nanomaterials, contributing to human and environmental safety while supporting the global goal of reducing animal testing within chemical regulations.

Introduction

In the European Union, manufactured or imported chemicals must be registered according to the provisions of the REACH regulation (Regulation (EC) No 1907/2006 on the registration, evaluation, authorisation and restriction of chemicals). REACH annexes VI to X specify the standard information requirements for registration purposes. In 2018, additional nano-specific data requirements were published for these annexes, implemented in Commission Regulation (EC) 2018/1881. Since January 2020, registrants of nanomaterials have been obliged to provide nano-specific information for compliant REACH registrations. According to Article 13(3) of the REACH regulation, tests conducted to fulfil these information requirements '*shall be conducted in accordance with the test methods laid down in a Commission Regulation or in accordance with other international test methods recognised by the Commission or the Agency as being appropriate*'. Based on this provision, newly generated experimental data for REACH registration purposes are generally accepted only if standardised and validated test guidelines are followed, as defined in the EU test methods regulation (Commission Regulation (EC) No 440/2008), which refers to test guidelines (TGs) of the Organisation for Economic Co-operation and Development (OECD) for many of the REACH-required tests. To date, nano-specific test guidelines are often missing or have only a limited applicability,[1–3] thus impeding the

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production of reliably accepted data. The situation is particularly challenging for purely carbonaceous nanomaterials, such as MWCNTs.

Relevant guidance documents of the European Chemicals Agency (ECHA) address carbonaceous nanomaterials only to a limited extent and provide no guidance on how to consider the specific properties of MWCNTs, which cause severe problems in tests required by REACH. These properties include extensive aggregation/agglomeration that lead to highly unstable dispersions and analytical problems in carbon containing media.[4–7] It is therefore not surprising that authorities recently published calls for identifying information gaps related to nanomaterials. For example, in 2024 and 2025, ECHA's European Union Observatory for Nanomaterials (EUON) issued calls for 'closing nanomaterials' information gaps: EUON welcomes new study proposals' and 'EUON calls for study proposals to address nanomaterials knowledge gaps in the EU'.[8,9]

Due to the limited availability of standardised and validated test guidelines for generating the obligatory nano-specific experimental information, registrants currently face several complex challenges. First, testing has to be adapted to some extent to address the properties of the specific nanomaterial (*e.g.*, poor dispersion stability) but guidance for such adaptations is often lacking for the specific TG that is requested by legal provisions. Second, while there is a wealth of academic literature on testing nanomaterials using a variety of approaches, their regulatory acceptance is often unclear to registrants. For example, a recent ECHA-sponsored report on existing methods for the detection and quantification of carbon-based nanomaterials in environmental and biological matrices[10] lists 57 methods and analytical techniques. This report also notes the lack of standardised protocols, making it uncertain whether '*resulting data are already accepted, or could soon be accepted, by authorities, and ECHA in particular, under legislation such as REACH*'.[10] This finding illustrates (a) the problems registrants face when selecting appropriate quantification techniques for studies required by REACH and (b) the uncertainty they have with respect to regulatory acceptance of their data. Third, registrants typically commission contract research organisations (CROs) with expertise in applying a specific TG. However, a CRO's experience in applying that method specifically to a nanomaterial (in this case MWCNTs) may be limited. For (eco)toxicological tests, REACH Article 13(4) further specifies that a CRO must be accredited according to principles of good laboratory practice (GLP), further limiting the number of CROs that can conduct the required test. In addition, a CRO may not have all analytical techniques available that could be envisaged. Since all techniques have their advantages and disadvantages for carbon-based nanomaterials, a '*multi-technique approach is often necessary*',[10] making it even more unlikely that a CRO with a specific OECD TG expertise will be equipped with all possible methods. Finally, generating data for REACH registration purposes often has to be performed within a certain time period due to legal deadlines and within a given budget, limiting the possibilities of applying a variety of different test conditions (*e.g.*, extending those prescribed in OECD TGs) or analytical techniques. The issues discussed above are interlinked. For example, a registrant is unlikely to spend extensive resources on several analytical techniques if their regulatory acceptance is questionable.

This study aims to share the knowledge gained through the practical application of TGs to fulfil REACH information requirements. It showcases the technical and practical challenges of generating data for real REACH registration dossiers, highlighting failures and successes that we encountered while generating new data, along with feasible adaptations to test guidelines that we believe are acceptable to regulatory bodies. It provides a realistic regulatory perspective that differs from studies conducted by research institutes or academia in several respects. For example, following the required TGs means that test concentrations in most cases cannot be chosen freely. Also, applying different analytical techniques is limited by availability as well as budget and time constraints, as discussed above. These constraints generally also prevent the repetition of studies under refined conditions after more experience has been gained. While these issues may be perceived as limitations, this study provides real-world insights that are of interest for stakeholders involved in data generation for REACH registration purposes and the broader scientific community interested in test methods for carbon-based nanomaterials.

As is evident from the above, this study is not a review of the existing literature for testing a specific endpoint, does not intend to validate TGs or analytical techniques for MWCNTs, nor does it aim to define new standard procedures; instead, it introduces innovative experimental approaches to overcome challenges of generating data for specific, difficult-to-assess endpoints of MWCNTs in a REACH context.

In this paper, we present our methods to overcome the burdens of non-compliance for human health endpoints under REACH annexes VII and VIII. These endpoints include *in vitro* serious eye damage/irritation, *in chemico* skin sensitisation, and *in vitro* gene mutation in mammalian cells. Challenges of data requirements for nanoparticle size properties are addressed in the companion article,[11] demonstrating that MWCNT nanoforms of this study consist of long, entangled bundles; additionally, a novel approach to determine the length of the individual tubes within a bundle was introduced. The other companion article[12] of this series highlights the challenges in generating regulatory acceptable data for environmental fate parameters by (a) describing the adaptations needed to demonstrate the absence of carbon dissolution against a ubiquitous carbon background, (b) showing that novel analytical techniques are needed due to rapid settling and agglomeration of MWCNTs in dispersion stability testing (ultimately demonstrating low dispersion stability), and (c) highlighting persisting problems in adsorption testing of MWCNTs.

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In vitro serious eye damage/irritation according to REACH annex VII, section 8.2.1

Since 2013, the REACH regulation has required the strict use of *in vitro* assays to fulfil data requirements related to serious eye damage/irritation. This approach aligns with the 3Rs principle (replacement, reduction, and refinement) for animal testing and promotes the advancement of new approach methodologies (NAMs), aiming to minimise the use of animals and their suffering, as implemented in, *e.g.*, EU Directive 2010/63/EU. However, available standardised *in vitro* guidelines are not specifically designed for nanomaterials. We applied the new OECD TG 492B 'reconstructed human cornea-like epithelium (RHCE) test method for eye hazard identification'[13] and successfully adapted the test protocol for the very light powder form of the investigated MWCNTs. We produced reliable and adequate *in vitro* data for assessing serious eye damage/irritation and demonstrated their suitability for the classification and labelling requirements of the CLP regulation (Regulation (EC) No 1272/2008).

In vitro/in chemico skin sensitisation according to REACH annex VII, section 8.3.1

Similarly, to serious eye damage/irritation, the REACH regulation requires the use of *in vitro* or *in chemico* assays to fulfil data requirements for skin sensitisation. However, available TGs are not specifically designed for nanomaterials in powder form. To cover the endpoint sufficiently, the OECD '2-out-of-3' (2o3) approach for chemical key events of skin sensitisation was applied. This methodology involves the selection of relevant test guidelines in a preliminary step.[14] After evaluating the applicability domain of the recommended *in chemico* tests, we selected the following two assays as they appeared most suitable for carbonaceous solid MWCNTs: the KeratinoSens™ assay according to OECD TG 442D[15] and the h-CLAT assay according to OECD TG 442E.[16] However, nano-specific recommendations are only available for the KeratinoSens™ assay (OECD No. 382 (ref. 17)). As MWCNTs exist as solid powder, the most challenging part of applying these guidelines was to achieving the obligatory stable test dispersions—'*i.e., a colloid or suspension in which the test chemical does not settle or separate from the solvent into different phases*'.[15,16] Consequently, we thoroughly investigated the solubility and dispersibility of our MWCNT nanoforms in various solvents to determine if the test material falls within the applicability domain of OECD TG 442D and TG 442E. Our ultimate goal was to produce reliable and adequate *in chemico* data for MWCNTs, thereby covering the skin sensitisation endpoint for both the REACH and CLP regulations.

In vitro gene mutation study in mammalian cells according to REACH annex VIII, section 8.4.3

The toxicological endpoint of *in vitro* gene mutation investigates a chemical's potential to induce gene mutations in mammalian cells, typically at the HPRT locus in V79 cells of the Chinese hamster. The HPRT assay is performed according to principles outlined in OECD TG 476 '*in vitro* mammalian cell gene mutation tests using the Hprt and xprt genes'.[18] For testing nanomaterials, the NANOGENOTOX dispersion protocol[19] offers guidance on the preparation of nanomaterial dispersions in exposure media. In principle, both the HPRT assay and the NANOGENOTOX dispersion protocol could be applied MWCNTs.

Materials

Multi-walled carbon nanotubes substance-specific information

K-Nanos. K-Nanos MWCNTs are elongated high-aspect-ratio MWCNTs that are aligned and entangled in bundle-type carbon agglomerates. Here, K-Nanos comprised three nanoforms, which were considered a 'set of similar nanoforms' based on REACH annex VI, amended by Commission Regulation (EU) 2018/1881: K-Nanos 100, K-Nanos 210, and K-Nanos 300. The purity of K-Nanos was >91%. The characteristics of constituent particles are as follows: the average individual carbon nanotube length and diameter ranged from 46.22–62.76 μm and 12.13–14.58 nm, respectively. Deriving from these values, the aspect ratio was 3789:1–4304:1. Average bundle length of MWCNTs ranged from 42.00–54.85 μm, while average bundle diameter from 2.460–3.211 μm. The specific surface area and the density were 200.8–247.7 $m^2\ g^{-1}$ and 1.76 g cm^{-3}, respectively. More nanoform-specific data can be found in SI Table S1.

Jenotubes. Similarly, Jenotubes MWCNTs are elongated high-aspect-ratio MWCNTs that are aligned and entangled in bundle-type carbon agglomerates. Here, Jenotubes comprised four nanoforms, which were considered as a 'set of similar nanoforms': Jenotube 6, Jenotube 8, Jenotube 10A and Jenotube 10B. The purity of Jenotubes was >97%. The characteristics of constituent particles are as follows: the average individual tube length and diameter ranged from 35.87–136.37 μm and 7.4–12.2 nm, respectively. Based on these values, the aspect ratio was calculated as 3261 : 1–16 961 : 1. Average bundle length of MWCNTs ranged from 35.6–129.8 μm, while average bundle diameter was in the range of 2.9–6.3 μm. Lastly, the surface area and the density were 217–644 $m^2\ g^{-1}$ and 2.29 g cm^{-3}, respectively. Nanoform-specific data can be found in SI Table S2.

Methods

In vitro serious eye damage/irritation

We analysed the eye irritation potential of K-Nanos 300 according to OECD TG 492B.[13] We followed the test protocol for the time-to-toxicity test method on solids (TTS), using the SkinEthic™ human corneal epithelium (HCE) *in vitro* assay.[20] The reconstructed human corneal epithelium (RHCE) model used in this study was the SkinEthic™ HCE/corneal epithelium (HCE/S/5), supplied as a standardised kit by EPISKIN (Lyon, France). This model is derived from immortalised HCE cells.

Cytotoxicity was expressed as reduced mitochondrial dehydrogenase activity measured by a decrease in purple

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formazan production from MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, CAS RN 298-93-1). Negative and positive controls were run in parallel with the treatment groups. A non-specific colouring control of living tissues (mixture of 10 mg test item per 90 μL *Aqua dest.* and 90 μL isopropanol) was performed to demonstrate that the test material did not exhibit any water-colouring potential.

At the start of the test, solid K-Nanos 300 MWCNTs were directly topically applied to the RHCE tissue (specific batches included 24-HCE-039 for the main experiment, 24-HCE-055_S for the killed tissue controls, and 24-HCE-041 for the viable tissue controls). Due to the low density of MWCNTs, the volume of required test material (corresponding to amounts of 80 ± 2 mg or 160 mg cm^{-2}) was too large to fit into the test well (SI Fig. S1). Therefore, a lower amount of test material (4.5 mg) was applied, which was nevertheless sufficient to cover the tissue surface entirely (SI Fig. S2). Since a lower amount than required by the TG was applied to the RHCE tissue, we performed non-specific MTT (NSMTT) reduction controls with K-Nanos 300 MWCNTs (4.8 mg) to rule out false-negative results. Cytotoxicity was measured after 30 and 120 minutes of exposure, as well as a 10 minute post-treatment period, and then compared to the concurrent negative controls. For each tissue, 2 × 200 μL aliquots of the extract were transferred into a 96-well plate. The optical density (OD) was measured in a microplate autoreader (Tecan Infinite M200 Pro, Tecan, Austria) using isopropanol as a blank. The mean tissue viability was calculated using UV-vis spectrophotometry at a wavelength of 570 nm (OD_{570}) (wavelength accuracy ≤ ±1.5 nm $\lambda > 315$ nm/≤ ±0.8 nm $\lambda \leq 315$ nm).

In vitro/*in chemico* skin sensitisation

We performed a solubility pre-study to investigate the solubility and dispersibility of K-Nanos MWCNTs in various solvent classes acceptable for the KeratinoSens™ and h-CLAT assays, as specified by OECD TG 442D[15] and 442E,[16] respectively. We analysed K-Nanos 300 MWCNTs in the following solvents: *Aqua dest.*, DMSO, ethanol, isopropanol, acetone, DMF, and 0.9% NaCl at test item concentrations of 1 and 500 mg mL^{-1} (abbreviation as per Table 1). The test material was added to each solvent immediately prior to evaluation.[21] For each test, K-Nanos 300 MWCNTs were separately weighed to achieve the targeted concentrations. The test suspensions were prepared as follows: (1) vortexing for 1 minute, (2) ultrasonication (80 W) and warming (37 °C) for 10 minutes (Table 1). Then, each dispersion was visually inspected and compared to identical aliquots of pure solvent. If a homogenous suspension was achieved in the first step, it was left at room temperature and again inspected after 2 hours. Suspension stability was qualitatively assessed with the unaided eye. The occurrence of visible precipitation or phase separation was monitored at the beginning (within minutes) and at the end of the test period (after two hours). In addition, a K-Nanos 300 MWCNT suspension was prepared at 20 mg mL^{-1} using the method proposed by Kim *et al.*[22] for OECD skin sensitisation test guidelines. These authors successfully prepared a stable stock dispersion of MWCNTs in distilled water by sonication at 100 W for 30 minutes, without providing details on the exact concentration of the prepared stock dispersion. The method was slightly adapted, and sonication was performed at 80 W for 30, 45, and 60 minutes. K-Nanos 300 MWCNTs were also directly added to the cell culture medium of both assays at the required maximum test concentrations. Following OECD TG 442D and OECD No. 382 for substances lacking a defined molecular weight, a *pro forma* molecular weight of 200 g mol^{-1} was assumed. Accordingly, the maximum test concentrations were set at 200 μg mL^{-1} (equivalent to 1000 μM) for the KeratinoSens™ assay and 1000 μg mL^{-1} (1 mg mL^{-1}) for the h-CLAT. The h-CLAT medium contained 10% FCS, and the KeratinoSens™ medium contained 1% FCS and 1%

Table 1 Summary of solubility pre-study results, including preparation of stock and test media and visual assessment of dispersion stability

Solvent/media	Test material conc.	Test condition	Dispersion stability
Aqua dest.	500 mg mL^{-1}	1 min vortexing	None[a,b]
Aqua dest.	1 mg mL^{-1}	1 min vortexing, 10 min ultrasonication (80 W, 37 °C)	None[a]
DMSO			None[a]
Ethanol			None[a]
Isopropanol			None[a]
Acetone			None[a]
DMF			None[a]
0.9% NaCl			Initially[c]
Aqua dest.	20 mg mL^{-1}	1 min vortexing, 60 min stepwise ultrasonication (80 W, 37 °C): 30 min, +15 min, + 15 min[d]	None[a]
h-CLAT (RPMI1640, 10% FCS)	1000 μg mL^{-1}	Direct addition to test media	Initially[c]
KeratinoSens™ (DMEM, 1% FCS, 1% DMSO)	200 μg mL^{-1}	Direct addition to test media	Initially[c]

[a] Visible sedimentation or phase separation occurred within minutes after preparation. [b] Volume of powder exceeded solvent volume. [c] Homogeneous suspension achieved initially, followed by visible sedimentation or phase separation after 2 hours. [d] Prolonged sonication adapted from Kim *et al.*[22] Abbreviation: Conc.: concentration, *Aqua dest.*: *Aqua destillata*, DMSO: dimethyl sulfoxide, DMF: dimethylformamide, RPMI1640: Gibco RPMI 1640 medium, FCS: fetal calf serum, DMEM: Dulbecco's modified Eagle medium.

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DMSO.[15,16,21,23] In principle, the KeratinoSens™ assay may be performed at even lower concentrations without any further specification given in the protocol; however, the protocol and relevant ECHA documents[24,25] further state that negative results obtained at concentrations <200 μg mL^{-1} (corresponding to 1000 μM) should be considered inconclusive.[21] As the primary aim was to generate REACH-relevant data, concentrations below 200 μg mL^{-1} were not tested, although such testing may provide additional insights.

In vitro gene mutation study in mammalian cells

The HPRT assay was performed with K-Nanos 100 and Jenotube 8 MWCNTs, following OECD TG 476.[18] While experimental conditions were similar for both nanoforms, the following detailed descriptions relate to the test with K-Nanos 100. The nanomaterial exposure medium was prepared according to the NANOGENOTOX dispersion protocol, and the maximum test concentration was 256 μg mL^{-1} (diluted from a 2.56 mg mL^{-1} stock dispersion).[19,26] A sterile 0.05% (w/v) bovine serum albumin (BSA)–water solution was used to prepare the test material dispersion for the exposure medium. To prepare a 2.56 mg mL^{-1} stock dispersion, 123.2 mg of the test material were pre-wetted with approx. 241 μL pure ethanol and then dispersed in 47.89 mL BSA–water (0.05% (w/v)). For the main experiment, the concentration range was spaced by a factor of approx. 2.5, resulting in concentrations of 0.4, 1.0, 2.6, 6.6, 16.4, 41.0, 102.4, and 256 μg mL^{-1}. While OECD TG 476[18] requires a minimum of four analysable concentrations, testing started with eight concentrations to overcome possible deviations in toxicity. To achieve a homogeneous dispersion, this mixture was ultrasonicated for approx. 1 minute at 200 W and approx. 10% amplitude. The plastic vial was cooled in an ice-water bath during sonication. The formulation was prepared freshly before treatment and used within 2 hours. Precipitation was visually evaluated with the unaided eye at the beginning and at the end of the treatment. Following a 4 hour treatment, cultures at 0.4, 2.6, and 16.4 μg mL^{-1} (without metabolic activation) and at 1.0, 2.6, and 16.4 μg mL^{-1} (with metabolic activation) were discontinued because the remaining five concentrations were sufficient to meet the OECD TG 476 requirement. Similarly, after a 24 hour treatment without metabolic activation, cultures at 1.0, 2.6, and 16.4 μg mL^{-1} were discontinued for the same reason. The overall treatment period was 4 hours (both with and without metabolic activation), and 24 hours (without metabolic activation).

Results

In vitro serious eye damage/irritation

The test material, K-Nanos 300 MWCNTs, did not induce any cytotoxicity to the reconstructed human cornea-like epithelium (RHCE) (SI Tables S3 and S4). NSMTT (non-specific MTT) controls further confirmed that K-Nanos 300 MWCNTs were unable to chemically reduce MTT to formazan. Therefore, any purple colouring observed in the test medium was entirely attributable to healthy cell activity. In detail, the mean % NSMTT reduction was ≤60% after both 30 and 120 minutes, indicating the test material was compatible with the test method, and the use of a lower amount of test material than demanded by the guideline did not negatively impact the test outcome. Specifically, % NSMTT was 0.1% and 0% after 30 and 120 minutes, respectively, and these values were subtracted from the results of the mean relative tissue viability of K-Nanos 300 (SI Tables S5 and S6). The NSMTT-corrected mean relative tissue viability was 103.4% after 30 minutes and 106.9% after 120 minutes (negative control set to 100%) (SI Tables S3 and S4).

The non-specific colouring control of living tissues showed no colouring effects that were detectable by the unaided eye. Since correction for non-specific colouring was not necessary, an additional control with killed tissues (to correct for a false-positive result) was not required. The results fulfilled the validity criteria of the test guideline (SI Table S7). K-Nanos 300 MWCNTs were assessed as not irritating or damaging to the eye, according to the criteria outlined in Table 5 of OECD TG 492B,[13] which states that the mean tissue viability must exceed >40% after 30 minutes and >60% after 120 minutes to meet the 'no category' for the SkinEthic™ HCE TTS (solids protocol).[13,20] Consequently, we concluded that classification of K-Nanos 300 MWCNTs for serious eye damage or irritation was not required according to UN GHS or the CLP regulation.

In vitro/*in chemico* skin sensitisation

K-Nanos 300 MWCNTs were not soluble and did not form any stable dispersion in the investigated solvents, *i.e.*, *Aqua dest.*, DMSO, ethanol, isopropanol, acetone, DMF and 0.9% NaCl, or in the test media of KeratinoSens™ and h-CLAT. Although initially a homogenous suspension could be achieved in both test media and the 0.9% NaCl solution, it was unstable after 2 hours. The volume of K-Nanos 300 MWCNT powder for the 500 mg mL^{-1} stock solution greatly exceeded the necessary solvent volume, a finding observed in the initial test run conducted with *Aqua dest.* This was attributed to the very low density of the test material and considered an inherent property of the test material. Since subsequent test runs at 1 mg mL^{-1} with the remaining solvents did not yield visible suspensions or dispersions either, further testing of 500 mg mL^{-1} stock solutions was omitted. Moreover, the additional step of prolonged sonication as described by Kim *et al.*[22] did not improve the suspension or dispersion of the test material, mirroring previous test results.

After this thorough investigation of various solvent types and test media of the relevant test guidelines, K-Nanos 300 MWCNTs were considered not applicable to testing in either the KeratinoSens™ or the h-CLAT assay under the given test conditions.

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In vitro gene mutation study in mammalian cells

No cytotoxicity was observed in the absence or presence of S9 mix after a 4 hour treatment in the main experiment, up to the highest test concentration. Following the 24 hour treatment, moderate cytotoxic effects were observed at 102.4 μg mL^{-1} and higher. No substantial and dose-dependent increase in mutation frequency was observed in any of the treatment groups (SI Tables S8–S10). Consequently, K-Nanos 100 MWCNTs were considered non-mutagenic to mammalian cells. Precipitation of the test material—characterised as a distinct cloudiness—was visually observed at all concentrations, except for the lowest test concentrations of 0.4 μg mL^{-1} and 0.1 μg mL^{-1} for the 4 hour treatment without metabolic activation (Table 2), indicating a limitation in dispersion stability. At no point was the concentration high enough to form a continuous surface film or macro-scale coarse particles that would cover the cell monolayer.

Appropriate reference mutagens, used as positive controls, induced a distinct increase in mutant colonies (SI Tables S8–S10). This demonstrated the sensitivity of the test system and the activity of the metabolic activation system. The validity criteria of the guideline were fulfilled.

Discussion

In vitro serious eye damage/irritation according to REACH annex VII, section 8.2.1

In vitro testing for serious eye damage/irritation can be conducted in various assays. None of the available OECD TGs for *in vitro* tests on serious eye damage/irritation comment on the feasibility of testing nanomaterials *per se*; however, they are generally applicable to solid test materials. In the following, we discuss two standard test guidelines with respect to their applicability to MWCNTs: OECD TG 492B 'reconstructed human cornea-like epithelium (RHCE) test method for eye hazard identification'[13] and OECD TG 437 'the bovine corneal opacity and permeability (BCOP) test'.[27] The RHCE assay is a stand-alone method capable of discriminating between the hazard sub-categories of serious eye damage/irritation according to GHS/CLP. In contrast, the BCOP assay requires additional testing if the test results are negative, addressing the need of further evaluation due to the high false negative rate for solids noted during test validation.[27] Since no potential of serious eye damage/irritation was expected for our test materials based on available *in vivo* data of a similar MWCNT nanoform, we chose to investigate the applicability and feasibility of the favourable RHCE assay (OECD TG 492B) to MWCNTs.

Applicability of OECD TG 492B to MWCNTs. OECD TG 492B[13] allows the direct application of neat solid substances, which can be directly applied in powder form. However, the guideline states: '*Caution should be used when testing solid chemicals with poor water solubility (<0.014 mg mL^{-1}) as they were frequently underpredicted by SkinEthic™ HCE TTS in the validation study*'.[13] The test method uses MTT to determine tissue cytotoxicity. According to Kroll *et al.*,[28] carbon nanoparticles interfered with MTT, leading to an apparent reduced cell viability. Although MTT-interfering chemicals are not generally excluded from the applicability domain of OECD TG 492B, appropriate controls must be included to account for any potential interference.[29] We examined whether MWCNTs reacted with MTT using non-specific MTT (NSMTT) reduction controls. The outcome was negative. The reported experimental results demonstrate that the *in vitro* method according to OECD TG 492B[13] was fully applicable for reliably assessing serious eye damage/irritation of MWCNTs.

Considerations for topical *vs.* suspension-based nanomaterial application. Topical application—or generally, any 'as such' application of a test material directly to a test system—extends the applicability domain of standard test guidelines to nanomaterials tested in powder form. Conversely, if a stable suspension or dispersion in a test medium is required for a successful test performance, these requirements exclude many poorly water-soluble solid nanomaterials, such as MWCNTs, from the applicability domain.

Concerning the topical application of a test material to a tissue, such as in the *in vitro* eye irritation assay following OECD TG 492B,[13] a lower amount of MWCNT test material was used than stipulated by the guideline. This deviation was necessary because the low density of MWCNTs led to an excessive volume of test material (SI Fig. S1). The deviation was not considered to negatively affect or bias the test outcome because we carefully ensured complete coverage of the tissue surface (SI Fig. S2), allowing full exposure to the test material. Consequently, the assay was deemed applicable to very light or low-density powder test materials.

Table 2 Occurrence of test material precipitation in the treatment groups of the HPRT assay

Test conc. [μg mL^{-1}]	−S9, 4 h	+S9, 4 h	−S9, 24 h
0.4	—	—	—
1.0	—	P	P
2.6	P	P	P
6.6	P	P	P
16.4	P	P	P
41.0	P	P	P
102.4	P	P	P
256.0	P	P	P

P: precipitation visible at the end of treatment. Conc.: concentration. Note, similar results were obtained for Jenotube 8 MWCNTs under analogous experimental conditions (SI Tables S11–S13).

In vitro/in chemico skin sensitisation according to REACH annex VII, section 8.3.1

Due to the inherent complexity of the skin sensitisation pathway, a single *in vitro* assay is insufficient to adequately assess this toxicological endpoint. Therefore, information on three key events of skin sensitisation is generally required from *in vitro/in chemico* tests to sufficiently address potential

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sensitising effects to the skin.[25] The ECHA document on skin sensitisation[25] specifies that information on three key events must be provided unless a conclusion on classification and risk assessment can be made using information from one or two key events. The document further notes that if significant sensitisation cannot be excluded, additional information (*in silico*, *in chemico*, *in vitro*) is needed. This means that a self-classification as a category 1 skin sensitiser without sub-categorisation does not fulfil the REACH information requirement.[25] Thus, REACH requires data that allow classification as GHS/CLP category 1A or 1B. Hence, data from assays addressing the three distinct key events of the adverse outcome pathway (AOP) for skin sensitisation, as defined by the relevant OECD document,[30] must be considered for such differentiation. The OECD '2o3' approach[31] uses two out of three assays to identify the skin sensitisation hazard of chemicals. The '2o3' approach is based on the three key events of the AOP: key event 1—covalent binding to proteins (*e.g.*, DPRA, TG 442C[32]); key event 2—keratinocyte activation (*e.g.*, KeratinoSens™, TG 442D[15]); and key event 3—dendritic cell activation (*e.g.*, h-CLAT, TG 442E[16]). This approach can already distinguish between GHS/CLP Cat. 1 and GHS/CLP 'not classified' chemicals and also provides scoring systems to differentiate between sub-categories 1A and 1B. Since the provisions of OECD TG 497 (ref. 31) are complex and an in-depth discussion is outside the scope of this article, the following sections focus only on assay applicability to MWCNTs.

OECD TG 442C addresses key event 1 and describes three *in chemico* test assays for investigating covalent binding to proteins, including the direct peptide reactivity assay (DPRA). In our view, these assays are unsuitable because they inherently require a certain solubility of the test material. Furthermore, OECD TG 442C provides only general statements on very poorly soluble test materials and offers no specific reference to nanomaterials. OECD TG 442D and 442E address key event 2 and 3, respectively. They both require a thorough solubility assessment of the test material, which we performed in the respective test media and solvents during the solubility pre-study. For testing key event 2 according to OECD TG 442D, we specifically evaluated the applicability of the KeratinoSens™ assay because nano-specific guidance is available (although not for MWCNTs in particular).[17] For testing key event 3 according to OECD TG 442E, we evaluated the applicability of the h-CLAT assay. The guideline's provisions for insoluble substances (*e.g.*, solubility investigation in saline or DMSO) are similar for all included assays, such as the GARD™skin assay. Crucially, assays for key event 3 (OECD TG 442E) provide no specific consideration of nanomaterials similar to OECD TG 442C (key event 1).

The results of the solubility pre-study of K-Nanos 300 MWCNTs (Table 1) were considered robust and sufficient for evaluating the applicability of the skin sensitisation assays. The assessment of dispersibility, which included evaluations by visual inspection (as stipulated by both guidelines), revealed that neither a solution nor stable suspension could be achieved in any of the investigated solvents and media. Since OECD TG 442D and TG 442E are intended neither to perform a quantitative or qualitative assessment of the suspension stability nor to describe the quality of the precipitate within the treatment media, no such in-depth assessment was performed during the solubility pre-study. Consequently, no additional descriptive qualifiers were used beyond the observation of whether visible precipitation or phase separation occurred at the beginning or end of the test period. We opted not to include a quantitative analysis in the assessment, as it was deemed too challenging due to the analytical hurdles associated with carbon-based, rapidly agglomerating MWCNTs (discussed in the companion article[12]). Although an initial, temporary homogenous suspension could be prepared by directly adding the test material to both test media (see Table 1), time-dependent sedimentation or phase separation occurred within 2 hours after preparation. We also considered applying the NANOGENOTOX protocol[19] for the preparation of dry powder nanomaterial dispersions, as referenced by the available nano-specific guidance[17] for OECD TG 442D (*i.e.*, pre-wetting in ethanol, followed by dispersion in 0.05% (w/v) bovine serum albumin (BSA)–water at a concentration of 2.56 mg mL^{-1} using probe sonication). Since this pre-dispersion step had already been performed with K-Nanos 100 and Jenotube 8 MWCNTs for the HPRT assay (referred to in the *in vitro* genotoxicity section), and precipitation occurred at test concentrations of 102.4 and 256.0 μg mL^{-1} after 4 or 24 hours, we concluded this pre-treatment would not enhance the dispersibility of our test material sufficiently for the required 48 hour incubation time for the KeratinoSens™ media. Neither could an improvement of dispersion stability be achieved by applying extended sonication to the test solution prepared in *Aqua dest.* (up to one hour, as recommended by Kim *et al.*[22]), leading to the conclusion that no stable dispersion could ultimately be prepared. Given that the incubation times for cell-based *in vitro* skin sensitisation assays range from 24 to 48 hours, the lack of dispersion stability compromises the reliable exposure to the prepared test concentrations (*e.g.*, nominal test concentration), potentially leading to false-positive or false-negative results. Consequently, the *in vitro* KeratinoSens™ and h-CLAT assays are not applicable to K-Nanos MWCNTs because a stable dispersion could not be prepared with any of the solvents or test media mentioned in the protocols. By inference, this conclusion is likely to apply to other *in vitro* skin sensitisation assays that require dissolved or stably dispersed test materials in solvents covered in Table 1, such as the GARD™skin assay. We cannot exclude that K-Nanos MWCNTs may behave differently in other solvents/media (*e.g.*, the media X-VIVO™ 15 of the IL-8 LUC assay not assessed here) but there is no profound evidence that this would be the case.

While OECD TG 442D does not specify a low concentration limit for testing, a negative result at

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concentrations below 200 μg mL^{-1} (corresponding to 1000 μM) should be considered inconclusive, as stated in the KeratinoSens™ protocol[21] and in relevant ECHA documents, including guidance R.7a.[24,25] There is strong evidence that MWCNTs are not sensitising to the skin based on available experimental *in vivo* studies for similar elongated MWCNT forms with high-aspect-ratio. As a negative *in chemico* result was anticipated, we did not pursue solubility pre-testing at concentrations below 200 μg mL^{-1}, because it would consequently yield data insufficient for a conclusive skin sensitisation assessment. In conclusion, skin sensitisation potential of K-Nanos MWCNT nanoforms cannot be assessed with currently available standardised *in vitro/in chemico* assays.

Practical implication of 'not applicable' or 'inconclusive'. The relevant ECHA guidance[24] states that in cases where *in chemico/in vitro* tests are not suitable for a test material, the *in vivo* local lymph node assay (LLNA) must be performed, as specified in REACH annex VII. As mentioned, performing *in vitro/in chemico* assays, even with limited applicability, would likely yield inconclusive results. Consequently, an *in vivo* study should be conducted. In our case, new testing was omitted since *in vivo* data were available for similar elongated, high-aspect-ratio MWCNTs, and the experimental *in vivo* data for skin sensitisation based on LLNA showed no sensitising potential. The data were read-across to the set of K-Nanos and Jenotubes to fulfil data requirements. We acknowledge that applying and justifying read-across data can be challenging, and grouping strategies are often not accepted because standards for acceptance are high regarding both the extent of justification and its written form, *e.g.*, lack of robust characterisation of the source substance, complex toxicokinetic profiles, or an insufficient similarity justification. However, we considered that in the present case, new animal testing should be avoided to adhere to the 3Rs principles of minimising animal suffering. In addition, the LLNA requires that solid test materials can be dissolved or suspended in suitable solvents/vehicles.[33] Consequently, the problems described above for *in vitro* tests may also apply to the LLNA. It is highly improbable that new scientific evidence would be generated. Available data provide sufficient weight of evidence to conclude that elongated, high-aspect-ratio MWCNT forms are not sensitising to the skin.

In vitro gene mutation study in mammalian cells according to REACH annex VIII, section 8.4.3

While not specifically designed for nanomaterials, the HPRT assay performed according to OECD TG 476 (ref. 18) is applicable to solids. OECD TG 476 states: '*for poorly soluble test chemicals that are not cytotoxic at concentrations below the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. […] At the concentration producing a precipitate, care should be taken to assure that the precipitate does not interfere with the conduct of the test*'.[18] It implies that relatively insoluble test materials should be evaluated up to or beyond their limit of solubility, at the highest concentration to be formulated as a solution or homogeneous dispersion in an appropriate solvent. Moreover, the NANOGENOTOX protocol provides standard operating procedures (SOPs) for preparing nanomaterial dispersions, including MWCNT nanoforms, in exposure media for genotoxicity testing.[19] The SOP was developed and finalised using six different forms of MWCNTs alongside several other nanomaterials, and specifically states that carbon nanotube powders are '*challenging to disperse due to the high degree of entanglement, aggregation, and variations in small sub-samples*'.[19] Therefore, careful observation of the dispersion stability of the test medium over time and potential sample-to-sample variations is recommended; however, in principle the HPRT assay is applicable to MWCNTs.

Sonication protocol and deviations. The NANOGENOTOX dispersion SOP for MWCNTs requires sonication for 16 minutes at 400 W and 10% amplitude. However, we deviated from the SOP by applying reduced ultrasonication for approximately 1 minute at 200 W and approximately 10% amplitude. This deviation was informed by the understanding that elongated MWCNT bundles tend to break during sonication, producing debris that may not reflect the effective particle size of the MWCNTs. Our initial aim was to perform low-energy ultrasonication (30 W for 10 minutes or 50 W for 5 minutes at 10% amplitude) to avoid breaking of bundles or tubes. However, this was technically not feasible at the CRO. Consequently, due to technical limitations, the ultrasonication power was set to the lowest technically possible energy input: a minimum of 200 W for 1 minute at 10% amplitude. Despite the potential for bundle and tube breakage, we did not expect relatively soft ultrasonication to negatively affect the results. In conclusion, the NANOGENOTOX dispersion protocol and the applied sonication power were sufficient to achieve the best possible dispersion stability under these technical conditions; the suspension remained sufficiently homogeneous for the 24 hour test duration, although precipitation was still observed at higher doses, which is in line with the test guideline.

Dispersion preparation and outcome. According to OECD TG 476, testing precipitating materials is permissible; indeed, for poorly soluble chemicals that lack cytotoxicity, it is required that the highest concentration analysed produces turbidity (cloudiness) that is visible to the unaided eye or with the aid of an inverted microscope at the end of the treatment.[18] According to the current OECD TG 476, at least four analysable concentrations must be used in two parallel cultures. Based on cytotoxicity and solubility some concentrations may not be continued at the time point of selection. This procedure compensates for unpredictable shifts in toxicity or solubility of the test substance that can interfere with the interpretation of the results. Following the

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NANOGENOTOX dispersion protocol, we (1) set the maximum test concentration at 256 μg mL^{-1}.[19,26] and (2) were able to prepare a homogenous test medium dispersion (using ethanol-pre-wetted K-Nanos 100 MWCNTs and sterile 0.05% (w/v) BSA–water solution) for the lowest test concentrations. Thus, we ensured the maximum required exposure to K-Nanos 100 MWCNTs—at which precipitation occurred—along with homogenously dispersed exposure conditions at the lowest test concentrations (Table 2).

The validity of the test outcome was confirmed as: (i) no relevant increase in mutant colony numbers was observed post-treatment; (ii) the mean mutant frequency (MF) for the solvent control and all evaluated concentrations remained within the 95% control limits of the historical solvent control data; (iii) linear regression analysis indicated no statistically significant dose–response trend; and (iv) although *t*-tests performed on specific concentrations showed MFs exceeding the concurrent solvent control, these values remained well within the 95% control limits of the historical data and lacked dose dependency (SI Tables S8–S10). Consequently, the criteria for non-mutagenicity were fulfilled, and the test material was classified as non-mutagenic. Similar results were obtained with Jenotube 8 MWCNTs under analogous experimental conditions (SI Tables S11–S13). In conclusion, OECD TG 476,[18] in conjunction with the NANOGENOTOX dispersion protocol,[19] can be successfully applied to MWCNTs yielding reliable and adequate experimental results for the HPRT assay.

Conclusion

We provided a comprehensive, state-of-the-art evaluation of the applicability and suitability of standard test guidelines for meeting regulatory requirements for carbon-based nanomaterials, derived from practical, material-specific experience. As noted in the introduction, generating data for REACH registration purposes differs from academic data generation in several respects, such as strict adherence to provisions in test guidelines (*e.g.*, with respect to test concentration), testing at GLP-accredited CROs, and significant time and budget constraints. While this may be perceived as a limitation, this study is unique precisely because it discusses challenges in applying the test guidelines for MWCNT testing against this background. While a few similar studies have been published, these do not—to the best of our knowledge—cover MWCNTs. For example, a recent study on *in vitro* skin sensitisation testing of inorganic nanomaterials following several OECD testing guidelines[34] focused on metal oxides and quartz.

The findings of our investigations demonstrate that adequate data may be generated only under two conditions: either solid nanomaterials can be applied as such (*e.g.*, OECD TG 492B), or nano-specific protocols are available to prepare stable dispersions (*e.g.*, the NANOGENOTOX dispersion protocol for OECD TG 476 testing). Our evaluation confirms that for skin sensitisation, K-Nanos MWCNTs cannot be assessed using current *in vitro* assays, necessitating further research to develop (1) suitable dispersion protocols for insoluble nanomaterials under the OECD test guidelines or (2) alternative assays.

If a standard test guideline is 'not applicable' to a test material or results are 'inconclusive', regulatory implications are difficult to predict. There are no guidelines or guarantees on what extent of deviation or adaptation is acceptable. Where current standardised assays fail, other new approach methodologies (NAMs)—including *in silico* approaches, emerging technologies, such as omics-based methods or high-throughput screening—still face limited acceptance in the regulatory context of chemical risk assessment.

With this research, we support compliance professionals, regulatory bodies, and researchers in closing information gaps related to *in vitro/in chemico* standard testing guidelines for assessing serious eye damage/irritation, skin sensitisation, and gene mutation in mammalian cells. This work contributes to the advancement of animal-free testing of human health endpoints for solid nanomaterials.

Author contributions

Marie-Léonie Bohlen was responsible for conceptualisation, writing – original draft preparation, validation, and supervision. She significantly contributed to writing – review & editing, and the investigation of the *in vivo/in chemico* assays. Jan Oltmanns made a major contribution by shaping the conceptualisation, methodology, and validation of the *in vivo/in chemico* approaches, and contributed to writing – review & editing. Hana Jo and Yeojin Lee contributed to writing – review, and project administration. Hyun Pyo Jeon contributed to funding acquisition.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of supplementary information (SI).

Supplementary information is available. See DOI: https://doi.org/10.1039/d5en01019e.

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