MACRAMÉ

Deliverable Report D2.8

Scientific Document to Support the Development of a Guidance Document for in vitro Inhalation Hazard Assessment of Advanced Materials

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[1] R= Document, report (excluding the periodic and final reports); DEM = Demonstrator, pilot, prototype, plan designs; DEC = Websites, patents filing, press & media actions, videos, etc.; DATA =Data sets, microdata, etc.; DMP = Data management plan; ETHICS = Deliverables related to ethics issues; SECURITY = Deliverables related to security issues; OTHER = Software, technical diagram, algorithms, models, etc.

[2] PU = Public, fully open, *e.g.* web (Deliverables flagged as public will be automatically published in CORDIS project's page); SEN = Sensitive, limited under the conditions of the Grant Agreement; Classified R-UE/EU-R = EU RESTRICTED under the Commission Decision No2015/444; Classified C-UE/EU-C = EU CONFIDENTIAL under the Commission Decision No2015/444; Classified S-UE/EU-S = EU SECRET under the Commission Decision No2015/444

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Acronyms Listed in this Document	
AdMas	Advanced Materials
AFM	Atomic Force Microscopy
ALI	Air-Liquid Interface
AMA	Alveolar Macrophage Assay
APS	Aerodynamic Particle Sizer
BaSO4	Barium Sulfate
BET	Brunauer-Emmett-Teller (method for specific surface area determination)
CD54	Cluster of Differentiation 54 (ICAM-1)
CD86	Cluster of Differentiation 86
CeO2	Cerium Oxide
CRM	Confocal Raman Microscopy
DLS	Dynamic Light Scattering
EC	European Commission
ELS	Electrophoretic Light Scattering
FCS	Fetal Calf Serum
FLG	Few-Layer Graphene
GD	Guidance Document
GIVIMP	Good *In Vitro* Method Practices
GO	Graphene Oxide
GRM	Graphene-Related Materials
H2O2	Hydrogen Peroxide
IL8	Interleukin-8
ILC	Inter-Laboratory Comparison
iPSC	induced Pluripotent Stem Cells
LA-ICP-MS	Laser Ablation Inductively Coupled Plasma Mass Spectrometry
LAL	Limulus Amebocyte Lysate (endotoxin assay)
LDH	Lactate Dehydrogenase
MMAD	Mass Median Aerodynamic Diameter
Mn2O3	Manganese(III) Oxide
MWCNT	Multi-Walled Carbon Nanotubes
NTA	Nanoparticle Tracking Analysis
OECD	Organisation for Economic Co-operation and Development
pHBE	Primary Human Bronchial Epithelial cells
SEM	Scanning Electron Microscopy
SMPS	Scanning Mobility Particle Sizer
SOP	Standard Operating Procedure

Acronyms Listed in this Document	
sQCM	Scanning Quartz Crystal Microbalance
TEER	Trans-Epithelial Electrical Resistance
TEM	Transmission Electron Microscopy
TiO2	Titanium Dioxide
TNF-A	Tumour Necrosis Factor alpha
ToF-SIMS	Time-of-Flight Secondary Ion Mass Spectrometry
TSLPR	Thymic Stromal Lymphopoietin Receptor
UC	Use-Case (UC1 to UC5)
WC-Co	Tungsten Carbide-Cobalt
WP	Work Package
XPS	X-ray Photoelectron Spectroscopy
ZnO	Zinc Oxide

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Executive Summary

Deliverable D2.8 on *'Scientific Document to Support the Development of a Guidance Document for in vitro Inhalation Hazard Assessment of Advanced Materials'* is the capstone scientific document of Work Package 2 (Task 2.5 - Hazard Assessment of AdMas in MACRAMÉ Use-Cases) of the MACRAMÉ Project (*Advanced Characterisation Methodologies to assess and predict the Health and Environmental Risks of Advanced Materials*, Grant Agreement No. 101092686). It provides the scientific foundation for the development of a harmonised Guidance Document (GD) for *in vitro* inhalation hazard assessment of advanced materials (AdMas), drawing on the collective experimental outputs of companion deliverables D2.2–D2.7 [3,4,5,6,7,8] and D2.9 [9] and the results generated across five representative industrial Use-Cases (UCs): UC1: Graphene oxide (GO) flakes in drinking-water filters, UC2: Few-layer graphene (FLG) in battery management systems (BMS), UC3: Graphene-related material (GRM)-bearing sprays, UC4: Carbon nanotubes (CNTs) in polymer foils for thermal applications (*e.g.* heated car seats), and UC5: PLGA (poly lactic-co-glycolic acid) (nano)particles -based inhalable antibiotics.

The document responds to a recognised and pressing regulatory gap. While OECD Guidance Document No. 39 (2018) provides a framework for inhalation toxicity studies, it remains primarily oriented towards *in vivo* animal-based testing and does not offer a validated, harmonised methodology for *in vitro* inhalation assessment of composite, life-cycle-transformed, or formulated AdMas. The present deliverable addresses this gap in a manner consistent with *OECD Good In Vitro Method Practices* (GIVIMP, 2025).

The scientific strategy is built around a structured, four-tier testing framework integrating physicochemical characterisation, aerosol generation, and biological endpoint assessment. The five Use-Cases covering graphene-related materials in composite matrices, consumer spray formulations, and dry powder systems provided a diverse and industrially relevant testing matrix, enabling systematic evaluation of the proposed workflow under realistic conditions.

Key scientific outcomes are as follows:

- Material characterisation: A minimum characterisation dataset has been defined, including primary size and morphology, surface chemistry, hydrodynamic diameter in biological media, BET surface area, endotoxin content, and for aerosol exposures, particle size distribution and mass median aerodynamic diameter (MMAD). Confocal Raman Microscopy (CRM) was identified as the method of choice for identification and spatial mapping of graphene-related materials in complex composite matrices, complementing XPS and AFM where applicable.
- Exposure systems: Four aerosol exposure platforms were evaluated: the Alveolar Macrophage Assay (AMA) under submerged conditions, Semi-ALI, Vitrocell® Cloud

[3] D2.2 - *Report on the SOPs developed by WP2* (R) (SEN) (M18)

[4] D2.3 - *Report: Performances & regulatory Readiness of SOPs for Aerosol Generation* (R) (SEN) (M18)

[5] D2.4 - *Report: Performances & regulatory Readiness of 'cell line'-based in-vitro assays* (R) (SEN) (M18)

[6] D2.5 - *Report: Performances & regulatory Readiness of advanced in-vitro Assays for Hazard Assessment* (R) (SEN) (M24)

[7] D2.6 - *Report: Performances & regulatory Readiness of ex-vivo assays* (R) (SEN) (M24)

[8] D2.7 - *Report: Performances of High-Resolution Imaging Methods* (R) (PU) (M34)

[9] D2.9 - *Report on Characterisation and Hazard Assessment in MACRAMÉ Use-Cases* (R) (SEN) (M40)

(including spray adaptation), Vitrocell® PowderX, and the fluidizer system. Each platform was found to be suited to specific material types and dose ranges, with complementary strengths across the Use-Cases. An overview of selection criteria is provided to guide future users.

- Biological testing: Property-outcome relationships were identified across the Use-Cases. Life-cycle transformation (abrasion, incineration) substantially altered the physicochemical and hazard profiles of materials. Carrier matrix effects were found to dominate biological responses in spray formulations (UC3), where the oil-based carrier drove stronger cytotoxic and immune activation responses than its silicone-based counterpart. Surface chemistry influenced cytokine profiles independently of cytotoxicity.
- Inter-laboratory comparison (ILC): The AMA was formally evaluated in an ILC across three independent laboratories. Seven of twelve tested particles were consistently classified as active or passive. All identified sources of variability, including FCS batch effects, TNFα kit sensitivity, and suspension preparation timing, are characterised and addressable through the harmonisation principles proposed in this document.
- Remaining gaps: No consensus dose metric currently exists for fibrous or high-surface-area AdMas. Long-term and repeat-exposure protocols are lacking. Quantitative detection of graphene-related materials in biological matrices remains technically challenging. These gaps define the priority research agenda for future guideline development.

Based on these outcomes, this document proposes the minimum testing battery and methodological requirements that a future harmonised GD for *in vitro* inhalation hazard assessment of AdMas should incorporate. These are further contextualised within the broader set of MACRAMÉ recommendations presented in Deliverable D5.4 - *Final Recommendations on future Needs for TGs & Standards Developments*.[10]

1 Introduction

1.1 Objective of the Document

Deliverable D2.8 is a scientific document intended to support the development of a harmonised Guidance Document for *in vitro* hazard assessment of advanced materials via the inhalation route. It synthesises the methodological developments, experimental results, and lessons learned across MACRAMÉ WP2 (Task 2.5 - *Hazard Assessment of AdMas in MACRAMÉ Use-Cases*). The document is not itself a guideline document but articulates the scientific basis that would support such a document and identifies the conditions under which proposed methods are valid and applicable. The document is also linked to the deliverable D5.4 [10] on final recommendations on needs for test guideline & standards developments which presents the final set of MACRAMÉ Informed Recommendations on the development and standardisation of test methods for Advanced Materials (AdMas). These recommendations arise from a structured, cross-partner collaborative process, drawing on the scientific outcomes of the MACRAMÉ work packages and broad stakeholder engagement.

[10] D5.4 - *Final Recommendations on Needs for Test Guideline & Standards Developments* (R) (PU) (M42)

1.2 Background and regulatory Context

The OECD Guidance Document No. 39 on Inhalation Toxicity Studies (second edition, 2018) provides guidance on the design and interpretation of inhalation studies and includes a section on manufactured nanomaterials. However, GD No. 39 remains primarily oriented towards animal-based *in vivo* studies and does not provide structured guidance for *in vitro* inhalation testing systems. The present document draws on this framework, particularly regarding aerosol characterisation and dosimetry, and extends it to the *in vitro* domain, in a manner consistent with OECD principles for *Good In Vitro Method Practices* (GIVIMP, OECD, 2025a).

A critical limitation in the current regulatory landscape is the absence of accepted, harmonised *in vitro* test methods for inhalation hazard assessment of AdMas. Existing frameworks were designed primarily for soluble chemical substances, and although nanomaterial-specific guidance has since emerged (*e.g.* the OECD WPMN guidance, the manufactured nanomaterials section of GD No. 39, OECD 2025b), these remain largely oriented towards *in vivo* testing and do not address *in vitro* inhalation assessment of composite or life-cycle-transformed AdMas. The MACRAMÉ Project addresses this gap by developing and validating *in vitro* methods across five Use-Cases.

1.3 Relationship with other WP2 Deliverables

D2.8 is the capstone scientific document of WP2 and should be read alongside: D2.2 (IBE) on basic SOPs to prepare AdMas for testing, D2.3 (BAuA) on SOPs for aerosol generation; D2.4 (RIVM) on cell line-based *in vitro* assays; D2.5 (Epithelix) on advanced *in vitro* assays; D2.6 (EMPA) on *ex vivo* assays; and D2.7 (IBE) on high-resolution imaging methods, and D2.9 (LIST) on the characterisation and hazard assessment of MACRAME UCs.

2 Overall Strategy for *in vitro* Hazard Assessment of AdMas

2.1 Hazard Assessment Challenges for AdMas

Advanced materials present a fundamentally different challenge for hazard assessment compared to conventional substances and, to some extent, classical nanoparticles. While they share certain difficulties with nanoparticles, such as dosimetry, agglomeration, and surface reactivity, AdMas add a further layer of complexity through composite-matrix effects and life-cycle-dependent transformations. Their biological activity depends on an interplay of size, shape, volume, surface chemistry, surface area, solubility, agglomeration state, and biopersistence, all of which may change substantially across life-cycle stages. Key challenges encountered in MACRAMÉ include: physicochemical heterogeneity requiring multi-technique characterisation; composite matrix effects that significantly modulate toxicological behaviour; life-cycle transformations (abrasion, incineration) that alter both physicochemical properties and biological activity; dosimetry challenges for non-spherical or aggregating particles. Finally, even though reference materials of single components such as graphene or multi-walled carbon nanotubes (MWCNTs) are available, well-characterised reference materials representing the multitude of composite AdMas classes are still missing.

2.2 Tiered Testing Strategy

The MACRAMÉ testing strategy is organised around four tiers: (i) physicochemical characterisation and dispersion protocol development; (ii) the alveolar macrophage assay (AMA, NR8383) for rapid cytotoxicity, lysosomal damage, oxidative stress, and inflammatory screening; (iii) cell line-based Air-Liquid-Interface (ALI) models for barrier integrity,

cytotoxicity, and immune activation under aerosol exposure; and (iv) advanced cell models (MucilAir, AlveolAir, ALIsens) and ex-vivo cultures (pHBE) for the highest physiological fidelity.

2.3 Integration of physicochemical Characterisation and biological Testing

Biological testing cannot be interpreted meaningfully without concurrent, material-specific physicochemical characterisation. Characterisation data serve to confirm material identity before testing, describe the material as delivered to cells, and support tracking within biological matrices to relate biological responses to effective doses, *i.e.* the dose cells are taking up or, at least, come into direct contact with. Integration of physicochemical and biological data should be treated as mandatory components of the testing workflow.

2.4 Workflow applied in MACRAMÉ

The experimental workflow applied across the five Use-Cases followed the tiered structure mentioned above. Each Use-Case material was characterised in pristine form and, where relevant, in mechanically abraded and/or incinerated forms, generated to produce respirable and/or inhalable particles necessary for inhalation *in vitro* models. Biological testing began with the AMA for materials amenable to submerged exposure (UC1, UC2, UC4, UC5), before advancing to ALI-based models. For UC3 spray formulations, aerosol-based exposure was prioritised from the outset using the Vitrocell® Cloud system, thus it was not included in the tier 1 testing with the AMA assay. MucilAir, AlveolAir and ALIsens models were used across several UCs for inhalation toxicity. In addition, the ALIsens co-culture model was used also across multiple UCs for immune activation endpoint assessment.

3 Material Characterisation Requirements

3.1 Recommended physicochemical Parameters

Based on the MACRAMÉ experience and alignment with existing OECD guidance, the following parameters are recommended prior to and during *in vitro* hazard assessment: primary particle/flake size and morphology (SEM, TEM, AFM); hydrodynamic diameter in the relevant biological medium (DLS or NTA); particle number concentration (NTA); specific surface area (BET); surface chemical composition (XPS, Raman); surface charge (zeta potential, measured by electrophoretic light scattering (ELS)) in biological media; chemical identity of the nanomaterial and carrier matrix; and, for aerosolised materials, particle size distribution (SMPS, APS), mass concentration, and MMAD. For life-cycle-transformed materials, full characterisation of the transformed form should be conducted.

3.2 Analytical Techniques

3.2.1 Raman Spectroscopy

Confocal Raman Microscopy (CRM) is the method of choice for identification and spatial mapping of graphene-related materials (GRM) in complex matrices. In MACRAMÉ, GRM such as graphene oxide (GO), few layer graphene (FLG), graphene, or MWCNT were successfully identified in polysulfone filters (UC1), epoxy resin (UC2), lubricant oils (UC3), and polyurethane foils (UC4). CRM was the only technique capable of directly detecting FLG within the epoxy composite in UC2 through 3D depth-profiling. ToF-SIMS and XPS, while complementary, cannot substitute CRM for direct GRM detection in such systems.

3.2.2 Surface and Morphology Analysis (XPS, AFM)

XPS is well-suited for quantitative surface chemistry analysis of well-defined, homogeneous nanomaterial preparations. In UC1, it provided a detailed characterisation of GO surface oxidation state. However, in complex composite matrices (UC2), overlapping C sp^2 and sp^3 signals and low GRM content precluded unambiguous graphitic carbon identification. AFM provided high-resolution topographic data on isolated GO flakes (UC1) but was inconclusive in the presence of ionic residues and complex surface chemistry (UC2). These techniques are most reliable for pristine, well-isolated nanomaterials and should be accompanied by a feasibility assessment for composite materials.

3.3 Impact of Material Properties on biological Outcomes

Across the MACRAMÉ Use-Cases, several property-outcome relationships were identified. Particle agglomeration state influenced macrophage uptake and necessitated size fractionation in some cases (UC2 abraded epoxy particles). Surface chemistry and functional groups affected cytokine profiles independently of cytotoxicity (*e.g.* TNFα induction by pristine FLG without cytotoxicity). Carrier matrix effects dominated responses in UC3, where the oil carrier drove stronger inflammatory and cytotoxic effects than the silicone-based counterpart. Life-cycle transformation substantially altered biological activity: in UC2 abrasion of FLG-epoxy composites reduced pro-inflammatory potential relative to the pristine composite, while incineration produced particles with distinct chemical signatures and altered hazard profiles.

3.4 Minimum Characterisation Requirements for *in vitro* Testing

As a minimum, the following dataset is required before *in vitro* testing: primary size/morphology (SEM or TEM), chemical identity (Raman and/or XPS), BET surface area (if sufficient material is available), endotoxin content (LAL assay), hydrodynamic diameter and polydispersity index in the dispersion medium, and agglomeration state over the exposure period. For ALI exposures, aerosol particle size distribution, mass concentration, MMAD, and temporal stability must also be reported.

4 Exposure Approaches

4.1 Overview

Four exposure platforms were evaluated in MACRAMÉ (Table 1), each suited to specific material forms and dose ranges. Selection should be guided by the physical state of the test material, the cell model used, and the target deposited dose. For all platforms, independently measured deposited doses should be reported alongside aerosol characterisation data (particle number/mass concentration, particle size distribution, and MMAD), and temporal stability of aerosol concentration and size distribution should be characterised for each new material.

Table 1: Overview of exposure systems tested within MACRAME.

Exposure Method	Pros	Cons	Best Used For
AMA / submerged conditions	Low cost	Restricted to particle dispersions	Initial screening to identify active materials
	Compatible with established dispersion protocols for serum free testing	Rat alveolar macrophage cell line NR8383	
	Highly motile cells		
	Uptake of particles from nano to ~10 µm		

Exposure Method	Pros	Cons	Best Used For
	Published prediction model for PSPs		
Submerged exposure of epithelial / barrier models (alveolar, bronchial inserts)	Human-relevant epithelial cells and co-cultures	Restricted to particles dispersible and stable in medium	Preliminary screening
	Compatible with barrier and multi-endpoint readouts (TEER, cytotoxicity, cytokines, uptake)	Not optimal for precise exposure Requires modelling of the dose which effectively is bioavailable for the cells	Particles that are still dispersible in medium
	Allows dose-response curves		
	Lower cost / higher throughput than ALI systems		
Semi-ALI	Simple setup	Uneven particle distribution (peripheral accumulation)	Preliminary screening
	Low cost	Residual apical medium (ALIsens)	Particles that are still dispersible in medium
	Can generate dose-response curves		
	Allows testing of different endpoints		
Vitrocell Cloud	Homogeneous and reproducible deposition	More complex and costly	Well-dispersible particles
	Realistic aerosol exposure	Longer preparation and exposure times	More accurate and uniform exposure
	Advanced system control		
Vitrocell Cloud (adapted for sprays exposure)	Enables realistic ALI exposure of liquid / consumer spray formulations	Inter-laboratory variability in deposition monitoring using sQCM	Consumer sprays formulation
		Gravimetric deposition used as primary dose metric	
	Can generate dose-response curves	Positional effects within the module (~2-fold dose variation between insert positions) must be controlled in study design	
	Allows testing of different endpoints		
Vitrocell PowderX	Direct powder exposure	High operational complexity	Poorly dispersible or hydrophobic particles
	- Suitable for poorly dispersible or dry particles	Expensive equipment	Dry powder aerosols
	No need for liquid suspension	Time-consuming	
Fluidizer	Low cost, flexible	Limited particle concentration	Tangled fibres
	High-power fibre disentanglement		

4.2 Semi-ALI Exposure

Semi-ALI exposure was implemented across all major lung models in MACRAMÉ as a practical and high-throughput-compatible approach. By applying a defined small apical volume of particle-containing medium, followed by evaporation-driven deposition onto the cell surface, Semi-ALI enabled the generation of dose-response curves and the assessment of multiple biological endpoints. It was particularly useful for preliminary screening and for particles that

remain dispersible in medium. In addition, the approach enabled confirmation of cellular uptake by SEM, 3D Raman mapping, and HR-TEM. However, Semi-ALI does not fully reproduce true aerosol deposition conditions and may result, depending on the model used (*e.g.* ALIsens) in uneven particle distribution, including peripheral accumulation, as well as residual apical medium. Consequently, it is less suitable for highly precise exposure scenarios, and the apical volume must be carefully optimised for each insert size.

4.3 Vitrocell® Cloud and Spray Adaptation

The Vitrocell® Cloud generates aerosols from liquid dispersions by ultrasonic nebulisation and was adapted in MACRAMÉ for consumer spray formulations (UC3). Variability was observed across laboratories where at Epithelix it was possible to follow material deposition with sQCM while at LIST the sQCM saturated after a single 1-second spray pulse for all formulations; gravimetric deposition was therefore used as the primary dose metric. In MucilAir bronchial cultures, silicone+graphene spray induced significant, dose-dependent reductions in cilia beating frequency and mucociliary clearance, and elevated IL-8 secretion, confirmed in two independent studies. In the ALIsens model, the graphene+oil formulation induced stronger cytotoxic effects and immune marker upregulation (CD54, CD86, TSLPr), demonstrating the dominant role of the carrier matrix. Positional effects within the Cloud module (approximately twofold dose variation between insert positions) should be systematically controlled in study design.

4.4 Vitrocell® PowderX Exposure System

The Vitrocell® PowderX system enables direct deposition of dry powders onto ALI inserts via a pressurised nozzle. In MACRAMÉ, it was used for the first time to expose the alveolar model comprising A549 cells at the ALI, the bronchial model developed by RIVM comprising Calu-3 and THP-1 differentiated macrophages and ALIsens co-culture to particles from the CML and GO Medica (UC1), with gravimetric deposition assessment. Significant dose-dependent decreases in cellular viability were observed across the different models. Vitrocell® PowderX is particularly suited to materials that cannot be aerosolised from liquid dispersions; its main limitation is its relatively low aerosolisation power, which is insufficient to disentangle or de-agglomerate cohesive or fibrous materials, for which the higher-power fluidizer is better suited.

4.5 Fluidizer System

The fluidizer generates dry powder aerosols via a vibro-fluidised. The released aerosol can be directed towards the Cyto-TP, a Vitrocell continuous flow cell exposure device with thermophoretic deposition enhancement. In MACRAMÉ it was applied to SAS, graphene, and GO, and MWCNT generating respirable aerosols (MMAD 3–5 µm) at concentrations suited to aerosol characterisation studies. Concentrations were estimated to be 4–10-fold lower than those achievable with PowderX or Vitrocell Cloud, limiting its utility for definitive ALI biological studies without further optimisation. The high power input enables the system to disentangle or de-agglomerate materials making it particularly well-suited to fibrous or high-aspect-ratio materials and serves as an accessible entry point for dry powder aerosol characterisation. In an innovative development during MACRAMÉ, MWCNT fibres were aerosolised using the fluidizer, and deposited by filtration onto cell culture inserts. The inserts were subsequently seeded with alveolar macrophages at ALI conditions. This approach was found to yield higher particle concentrations; however, further research is ongoing to explore its full potential.

5 Inter-Laboratory Harmonisation and Method Robustness

5.1 Rationale and Design

Regulatory acceptance of an *in vitro* method requires demonstration of reproducible results across independent laboratories. An inter-laboratory comparison (ILC) was conducted for the AMA as one of the most mature method in the WP2 toolbox. Three laboratories participated: IBE (Lead), BASF SE (Lab1, GLP-experienced industrial laboratory), and LIST (Lab2, academic research laboratory). Results have been submitted for publication (Tha et al. 2026, Frontiers in Toxicology).

5.2 Outcomes and Sources of Variability

Seven out of twelve tested particles were correctly and consistently classified as 'active' or 'passive' by all three laboratories using the Wiemann et al. (2016) two-out-of-four decision rule. Inconsistencies were observed for four borderline or interference-prone particles (BaSO4, ZnO, TiO2, CeO2). The principal sources of variability identified were: FCS batch effects on basal LDH (the most common cause of assay invalidity); inter-laboratory differences in TNFα kit sensitivity and detection range; differences in the timing of particle suspension preparation relative to exposure (critical for ionically active materials such as ZnO); and in-house preparation of GLU and H2O2 reagents introducing additional variability not present in kit-based assays.

5.3 Harmonisation Principles

Based on the ILC, the following harmonisation principles are proposed: provision of complete, unambiguous SOPs with pre-defined acceptance criteria for all quality control parameters; use of a common, pre-screened FCS batch; specification of a single commercial TNFα kit with documented sensitivity; centralised or protocol-controlled particle suspension preparation with a defined acceptable time window; centralised training at the Lead Laboratory; and pre-specified statistical decision criteria. These principles apply to the AMA and, by extension, to other *in vitro* inhalation hazard assessment methods undergoing inter-laboratory validation. Importantly, no unidentified sources of variability remained, all principal contributors being characterised and addressable through harmonisation.

6 Recommended Workflow for *in vitro* Inhalation Hazard Assessment

6.1 Selection of Exposure Systems

Dispersible particles should be tested with the AMA up to concentrations of 180 µg/mL. This concentration may lead to a maximum mean cell burden of up to 120 pg per alveolar macrophage, which is a reasonable upper effective dose for alveolar macrophages *in vivo* (Pauluhn et al., 2009). Size fractionation of particles and different dispersion protocols are applicable. Dry powders poorly soluble in water should be tested with Vitrocell® PowderX (preferred for reproducible deposition at 10–50 µg/cm²) or the fluidizer (best for tangled fibres). Liquid nanomaterial dispersions are best tested with the Vitrocell® Cloud for doses up to ~10 µg/cm², or semi-ALI for higher doses; nebulisation stability must be verified in advance. Consumer spray formulations require the Vitrocell Cloud adapted for spray exposure, with gravimetric dose assessment.

6.2 Biological Model

All models are complementary and provide different types of information. The AMA (NR8383) is used for initial particle screening; it is not applicable to spray formulations or fibrous materials without adaptation. Cell line-based ALI models (Calu-3/dTHP-1, alveolar A549 monolayer, ALIsens) provide initial information on barrier integrity and immune activation. Primary cell models (MucilAir, AlveolAir) provide the highest fidelity for bronchial and alveolar endpoints and are suited to repeat-exposure protocols. Ex-vivo pHBE cultures offer fully differentiated bronchial epithelia and are particularly useful for ciliotoxicity and mucociliary clearance assessment.

6.3 Endpoint Selection

The minimum endpoint panel for AdMas inhalation hazard assessment should include: cytotoxicity (LDH, and mitochondrial activity for ALI models); barrier integrity (TEER); inflammation (IL-8, TNFα); and oxidative stress (H2O2 for AMA; ROS assays for advanced models). For bronchial models exposed to spray formulations, cilia beating frequency and mucociliary clearance are recommended given the potential for carrier-mediated ciliotoxicity. Immune activation markers (CD54, CD86, TSLPr) should be included for the ALIsens model. Additional endpoints, proteomics, epigenetics, genotoxicity, may be considered for Tier 4 assessment of priority materials.

7 Limitations and remaining Knowledge Gaps

7.1 Technical Limitations

Several technical limitations must be acknowledged: deposited dose in ALI experiments is typically estimated gravimetrically rather than measured directly at the cell surface, introducing uncertainty for aggregate materials; the fluidizer generated aerosol concentrations approximately 10-fold below those required for unambiguous testing for biological effects without further optimisation; spray formulations could not be tested in standard submerged formats, preventing direct comparison with AMA outputs; and highly absorbing carbon materials (graphene, MWCNT) can interfere with fluorescence-based endpoint assays, requiring dedicated interference controls.

7.2 Methodological Gaps

No consensus dose metric exists for different AdMas classes; mass-based or volume-based metrics as used in the AMA may not be appropriate for fibrous or high-surface-area materials. The Wiemann et al. (2016) AMA prediction model was developed for poorly soluble nanoparticles and requires extension to fibrous materials, spray formulations, and soluble substances. Long-term and repeated exposure protocols remain lacking for materials with suspected biopersistence. Quantitative detection of GRM in biological matrices by Raman is not yet established, and alternative methods such as thermography still need to be explored (Steinmetz et al. 2020). LA-ICP-MS quantification of ingested materials such as CeO2, Mn2O3 and WC-Co were established for macrophages spin-dried on carriers (D2.7) but are technically challenging and not suitable for carbon materials.

7.3 Transferability and future Research Priorities

Advanced primary cell models and *ex vivo* cultures depend on human donor tissue and require specific expertise. In addition, dedicated equipment (Vitrocell® Cloud, Vitrocell® PowderX) is

needed, which is commercially available but not yet widely implemented in testing laboratories. Priority future research needs include: development of validated quantitative dosimetry methods for ALI aerosol exposures; expansion of the reference material library for AdMas ILC activities; and establishment of a read-across framework integrating physicochemical and *in vitro* hazard data to support grouping and regulatory decision-making.

8 Proposed Elements for a future Guidance Document

The prospective GD should cover poorly soluble engineered nanomaterials in dry powder form, nanomaterials embedded in composite matrices, formulated products containing nanomaterials in liquid carrier systems, and life-cycle-transformed forms. Materials with very high solubility, extreme pH reactivity, or strong optical interference may require method adaptation. The minimum testing battery should comprise: physicochemical characterisation (as specified in Section 3.4); Tier 2 AMA screening with the established prediction model (Wiemann *et al.* 2016, Tha *et al.* 2026) to differentiate between active and passive materials; Tier 3 ALI exposure methods covering cytotoxicity, metabolic activity, TEER, and/or IL-8 and cell surface marker expression as minimum endpoints.

9 Bibliography

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