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Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation Part 1: Final Project Report and Recommendations with Methodology to Prioritise Key Events (KEs) Relevant for Manufactured Nanomaterials

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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

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ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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Table of Contents

Foreword

1. The OECD has a key role in standardising methodologies for hazard testing and assessment and promoting best practices for the safe use of chemicals and the protection of human health and the environment. The OECD has established a number of programmes addressing different aspects of chemical safety enabling a sound harmonised approach for industrial chemical management. The Working Party on Manufactured Nanomaterials (WPMN) was established to ensure that the approaches for hazard, exposure and risk assessment for manufactured nanomaterials are properly integrated in the assessment of chemicals and aligned with the high quality, science-based and internationally harmonized tools developed by the OECD Chemicals Programme.

2. With this in mind, the WPMN launched the project *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*. The objective is to contribute to the future development and application of AOPs for MN regulatory decision making, by following the principles established by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). The outcomes of the project are presented in three complementary documents addressing:

- The scope of the project, its development and summary of the main conclusions. The document includes a methodology to identify, analyse and evaluate existing nanotoxicology literature with the objective to prioritize Key Events (KEs) relevant for MNs;
- A case study focused on a specific Key Event (KE) in the inflammation pathway to analyse the empirical evidence and contribute to the development of a knowledge base to inform AOP development and assessment for MNs; and
- The report from the OECD workshop *Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation*, which was organised in collaboration with the European Union (EU) Horizon 2020 projects *SmartNanoTox* and *Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment* (PATROLS). At this workshop, stakeholders had an opportunity to provide feedback on the methodology proposed, as well as on the case study, and to reach consensus on areas that could be further explored in the short, medium and long term.

3. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

Executive Summary

4. Adverse Outcome Pathways (AOPs) are conceptual frameworks that link key events (KEs) resulting from chemical or material exposure to adverse health or environmental impacts (Adverse Outcomes; AOs) important for evaluating safety. AOPs are designed as frameworks to organize toxicological information and offer a systematic and mechanistic approach to develop, assess, use, and interpret alternative testing strategies as part of Integrated Approaches to Testing and Assessment (IATA). Applied to IATA, AOPs are ultimately expected to reduce reliance on animal testing and are anticipated to be useful in risk assessment by linking alternative testing to health and environmental effects of nanomaterials and other emerging substances in a more systematic way.

5. To build on significant advances in AOP development over the last decade, and investigate the use of AOPs for manufactured nanomaterial (MN) safety assessments and decision making, the OECD's Nanosafety Programme initiated the project "*Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorisation* (NanoAOP project)". Its goal was to establish an approach to advance future AOP development that has the most significant potential to inform future categorisation and risk assessments of MNs using existing and available nanotoxicity literature. This goal was accomplished through three related objectives.

6. As a first step, there was a review of existing nanotoxicology literature to identify critical KEs for MNs; which allowed the selection of ***tissue injury*** as KE case study. Once the KE was selected, experts gathered and analysed critical information relevant to evaluate the use of the AOP framework for the risk assessment of MNs. As such, the project first demonstrated (i) a systematic process for mining the nanotoxicity literature to identify potential KEs relevant for MNs; and (ii) a strategy to prioritize identified potential KEs for development (Objective 1). Secondly, the project aimed at testing a methodology for developing prioritized KEs relevant for MNs from evidence gathered from the literature, *i.e.,* establish the plausibility of the KE, its essentiality in driving the AO, and compiling the empirical evidence to support the KE (Objective 2). To this end, a case study was developed on tissue injury[1]. Finally, the results of the first steps were presented at two workshops convened in 2018 (Halappanavar, 2019; Ede, 2020) and 2019[2] (OECD, 2018; 2019) to get (i) feedback on the approaches and methodologies developed in the NanoAOP project, and (ii) input on the status, use, and future needs for use of AOPs in the risk assessment of MNs[3].

7. Workshop participants agreed on a number of recommendations addressing current issues in development, application and acceptance of the AOP framework and related testing tools for use in MN decision making. Key recommendations include: ensuring MN-relevant considerations are accounted for in future AOP development; establishing test methods and protocols and verifying their predictive capability thus enabling use of AOPs for MN decision making; identifying, promoting and developing guidance on the current screening level-applications of AOPs that can be used for MN decision making; and promoting

[1] See document "Part2: Case Study on Tissue Injury" [ENV/JM/MONO(2020)34]

[2] One workshop was organised by Canada with the HORIZON 2020 SmartNanoTox project and the other one is the OECD workshop

[3] This is made available in document, Part3: Workshop Report and Recommendations [ENV/JM/MONO(2020)35]

reliable and quantitative MN data development to ensure MN-relevant AOPs can be developed and used in decision making. [4]

[4] The detail conclusions can be found in the document, Part3: Workshop Report and Recommendations [ENV/JM/MONO(2020)35].

1 Introduction

8. MNs are a diverse class of chemical substances with numerous potential applications that offer many benefits to society. However, their commercial adoption is hindered by the lack of accepted and efficient methods to evaluate the safety of the growing number, diversity, and complexity of MNs entering the market. The current approaches require individual risk assessments for each MN using traditional animal testing methods, which is both time- and cost-intensive and undesirable for ethical reasons. The OECD has a dedicated programme to ensure the development of standard methods for safety testing of chemicals, which includes nanomaterials. This programme ensures the methods meet regulatory needs, reflect scientific progress, address animal welfare aspects, and improve cost-effectiveness of test methods.[5] Recently, efforts such as the Malta Initiative are working to update or amend OECD Test Guidelines to include MN-specific considerations in safety testing.[6] In addition to this work, OECD has a dedicated programme on the development of Adverse Outcome Pathways[7]. AOPs are conceptual frameworks that link key biological events resulting from chemical or material exposure to adverse health or environmental impacts important for evaluating safety. AOPs are designed as frameworks to organize toxicological information and offer a systematic and mechanistic approach to develop, assess, use, and interpret alternative testing strategies as part of an IATA. AOPs are ultimately expected to reduce reliance on animal testing and allow categorisation and grouping of MNs. As AOPs represent mechanistic frameworks outlining biological processes that begin with Molecular Initiating Events (MIEs) and lead to an adverse outcome (AO), they are by definition not substance-specific or event-specific.

9. Significant advances in AOP development over the last decade have mainly focused on known toxicological mechanisms of chemicals and did not consider MN-specific mechanisms. A number of challenges remain to operationalize the AOP framework, and to translate these advances into established mechanistic pathways for use in MN safety assessment and decision making. There is a growing opportunity in: (i) supporting the future development of AOPs that capture toxicological mechanisms and contain KEs relevant for MNs; (ii) evaluating the use of the AOP framework as part of an IATA for MNs; and (iii) identifying how AOPs can be adopted for real-world applications in risk assessment and decision making for MNs.

[5] See: https://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm

[6] See: https://www.nanosafetycluster.eu/international-cooperation/the-malta-initiative/

[7] See: https://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

2 Scope

10. In 2016 the OECD WPMN included in its programme of work the project titled, “Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorisation” (NanoAOP project). The overall goal of this project is to develop a case study and apply the methodology created to highlight and support the development of future AOPs that have the greatest potential to inform future categorisation and risk assessments of MNs. This goal is accomplished through three main Objectives:

- identify a systematic process to advance future AOP development relevant for MNs by searching and mining existing and available nanotoxicology literature to identify and prioritize KEs relevant for MNs. (**Objective 1)**
- identify a methodology, through a case study approach, for developing KEs relevant for MNs, from evidence gathered from the literature. (**Objective 2)**
- gather expert feedback on (i) the approaches and methodologies developed under this project and (ii) the current status, use and future needs of AOPs relevant to MNs in support of their use in risk assessment and decision making.(**Objective 3)**

11. In order to move forward, the NanoAOP project decided to develop a case study and agreed on a specific KE in the inflammation pathway as: (i) there is a substantial literature base examining the inflammation processes for MNs to build the proposed method and case study; and (ii) inflammation is an identified KE in many AOPs and precursor to several AOs relevant to MNs (e.g. Halappanavar, 2019; Ede et al., 2020). It is important to note that the focus was to propose a methodology for identifying and developing specific KEs using the existing nanotoxicology literature to inform future MN risk assessment, following the principles used by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Therefore, the purpose was not to develop a full AOP for a specific AO induced by MNs.

3 Criteria and methods to identify and prioritize potential key Events (KEs)

12. In line with the first objective of the project, criteria and methods were developed to identify and prioritize potential KEs from the nanotoxicity literature for case study development. This work is described in detail in Halappanavar et al. 2019, and summarized here.

13. The analysis used a database of nanotoxicity literature developed by the Swiss Small and Medium enterprise (SME) NanoCASE GMBH, with the financial support of the Swiss Federal Office of Public Health and the German Chemicals Industry Association (VCI). The Swiss-VCI database includes a subset of 11,000 nanotoxicology studies published between 2000 and 2013. For each study, the database organizes the types of MNs investigated and the assays, endpoints and toxic effects reported. To identify potential inflammation-associated KEs, the studies selected were those reporting specifically on inflammation. This resulted in 191 publications spanning ~60 different endpoints for 45 different MNs. Analysis of these data identified numerous biological events that are reported to occur following MN exposure, are associated with inflammation, and represent potential MN-relevant KEs in an AOP.

14. As a second step, a prioritization strategy was developed in order to rank the identified potential KEs for further development. KEs were assessed with three main criteria to ensure the selected KE is: (i) plausible; (ii) measurable; and (iii) potentially relevant for regulatory considerations. Potential KEs that occurred post-inflammation were chosen as they were more closely linked to a specific AO (e.g. fibrosis). On the other hand, those potential KEs that occurred acutely after exposure, and were transitory or reversible, were avoided in the case study selection. Accounting for these considerations, tissue injury, defined as damage to tissues involving structural and/or functional changes, was selected as the potential KE to serve as a case study.

Outcomes

15. As summarised above, Halappanavar et al. 2019 outlines the criteria and a method to identify, select, and assess KEs; assess the database to generate a list of potential KEs in the inflammation pathway; and proposed a KE from this list to serve as a case study. Efforts included developing minimum study quality criteria, such as formal metrics to evaluate literature in the database in order to ensure data quality and consistency. These are summarised in Part 2: Case Study on Tissue Injury Appendix 1[8].

[8] Part2: Case Study on Tissue Injury [ENV/JM/MONO(2020)34]

4 Developing Key Events (KEs) for MNs

16. The second step was to outline a methodology, through a case study approach, for developing KEs relevant for MNs, using information and data gathered from the literature. This work is described in more detail in the document, Part 2: Case Study on Tissue Injury[9], and summarized here.

17. The methodology includes an approach to further develop a database (NanoAOP database), complete a literature search and study quality evaluation to populate the database, with the subsequent use of that database to gather evidence: (i) for the occurrence of a KE following MN exposure and (ii) to support its development as a future KE in an AOP, according to EAGMST principles.

18. The Swiss-VCI database (mentioned in Section 3) was updated to include literature through 2017.[10] With the update of the database, additional endpoints for evaluating the tissue injury KE were included. The subsequent literature search and quality evaluation identified a total of 126 new peer-reviewed papers. The resulting database contained 245 publications, consisting of 294 studies, covering seven MNs with many variants (e.g. uncoated or coated, positively or negatively charged, different shapes etc.), and a total of 485 parameters related to the tissue injury KE. The methodology for developing and populating the database is outlined in the document, Part2: Case Study on Tissue Injury appendix[11].

19. The NanoAOP database was the tool used to analyse whether tissue injury is a KE relevant for MN-induced adverse effects. The analysis found that in addition to inflammation, events related to oxidative stress and cytotoxicity were the three most commonly assessed and reported biological events following MN exposure with a direct inference to tissue injury. Since they were assessed in parallel with inflammation, the mechanism reflects the interplay between the three.These frequently reported biological events are interpreted as upstream KEs to describe the salient features of 'tissue injury'. The NanoAOP database demonstrates that each of the upstream KEs is measurable and that the same three upstream KEs can be measured irrespective of the tissue type in both *in vivo* and *in vitro* models. Analysis of the NanoAOP database identified the various *in vitro* endpoints, methods and assays used to measure the upstream KEs for tissue injury. Together, these data facilitated the development of tissue injury as a KE relevant for MNs as it is both a measurable and observed biological event following MN exposure that is essential for toxicity, thus meeting all the requirements for KEs according to the EAGMST principles.

[9] Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation, Part2: Case Study on Tissue Injury, ENV/JM/MONO(2020)34. This cote will be replaced before its publication

[10] This effort was funded via the Horizon 2020 NanoCommons research infrastructure for nanosafety.

[11] Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation, Part2: Case Study on Tissue Injury, ENV/JM/MONO(2020)34

5 Approaches and methodologies related to AOPs to support MNs risk assessment and decision making

20. Two workshops addressing AOPs for MN, provided useful information for the completion of this project. The first workshop Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation was held in 2018 as part of the 9th International Conference on Nanotoxicology (NanoTox) in Neuss, Germany, was co-organised by NanoAOP project partners and the Horizon 2020 SmartNanoTox[12] project, coordinated by Vireo Advisors, with support from NanoCASE,[13] the Swiss Federal Office of Public Health (Switzerland) and Canada. The second event was the OECD WPMN Workshop on "Advancing Adverse Outcome Pathway (NanoAOP) Development for Nanomaterials Risk Assessment and Categorisation" (September 2019).[14] This workshop discussed the recommendations from the first workshop, together with the results of the case study.

NanoTox 2018 Workshop.

21. Hosted in collaboration with the Horizon 2020 SmartNanoTox project, and with support from NanoCASE, the Swiss Federal Office of Public Health (Switzerland) and Health Canada (Canada), the workshop discussed the criteria and methods to identify and prioritize potential KEs (Halappanavar *et al.*, 2019). Two panels were convened. The first panel of toxicologists deliberated on tissue injury as a KE of regulatory relevance and, the technical, scientific and research questions surrounding AOPs, including feedback on the NanoAOP project and initial approach. The panel generally agreed that tissue injury is valid as an important KE for case study development in the project and offered feedback on the project approach and future direction. The second panel of risk assessors and regulatory decision makers discussed the use of AOPs for MN risk assessment. Many experts agreed that AOPs are useful tools for MN risk assessment including hazard identification, development of alternative testing strategies, making testing more efficient, and in grouping, categorisation and read-across efforts. However, the panel also highlighted several challenges that need to be overcome to adopt AOPs as risk assessment tools. A summary, major findings and recommendations of the external NanoTox expert workshop are published (Halappanavar et al., 2019).

[12] See http://www.smartnanotox.eu/

[13] See https://nanocase.com/

[14] The report is available as document, Part3: Workshop Report and Recommendations ENV/JM/MONO(2020)35.

OECD 2019 Workshop

22. The OECD workshop was hosted in cooperation with the European Horizon 2020 projects SmartNanoTox and PATROLS. Morning plenary presentations outlined the results and outcomes from NanoAOP project Objectives 1 and 2 including the literature evaluation, database development, identification of potential KEs, and tissue injury case study, for expert feedback. Potential next steps for the NanoAOP project, as well as current limitations, were discussed with workshop participants. Two additional plenaries presented the results from related projects, SmartNanoTox and PATROLS, working to further the development and adoption of AOPs for MN risk assessment. Presentations included recommended actions to advance the development and application of MN-relevant AOPs generated at the 2018 workshop. Expert feedback from discussions held as part of the OECD workshop further refined the general recommendations and outline short-, medium- and long-term actions to achieve them (Ede, et al., 2020).

23. The detailed discussion is available in the document, Part3: Work shop Report and Recommendations workshop report [ENV/JM/MONO(2020)35], and summarized below.

24. Experts generally agree that the approach demonstrated in the NanoAOP project is useful for future development of MN-relevant AOPs. Feedback included: developing guidance to address identified limitations of the currently available MN literature; optimizing the database structure and using additional data sources for KE development; and expanding the methodology to include key event relationship (KER) and molecular initiating event (MIE) development. In discussions on the use of the AOP framework for decision making about the safety of MNs, experts agreed that currently the AOP framework can be used for: (i) ranking and prioritising MNs; (ii) identifying critical KEs to develop alternative testing strategies for; (iii) product development as part of a safer manufacturing approach; and (iv) together with 'omics' strategies, AOPs can be used to propose testing that could be predictive of AOs. However, there are also limitations that need to be addressed before AOPs can be used as a quantitative risk assessment tool for regulatory decision making. Limitations to be addressed include a lack of reliable and quantitative MN data for AOP development; addressing considerations of exposure; a need for guidance on the use of AOPs for quantitative risk assessment and decision making; and a lack of accepted alternative testing methods and models to use as part of an IATA.

25. Experts discussed limitations hampering the use of available MN literature for AOP development including: uncertainty arising from the different exposure conditions used between studies; different models used in each study (e.g. assays, cell lines, etc.); consideration of MN dispersion; the general lack of physical and chemical characterisation of MNs (especially with earlier studies); and others. Guidance is needed on whether and how to use these data for risk assessment purposes, including future AOP development. The field would also benefit from guidance outlining the types of data that would be relevant for regulatory decision making to guide future testing and reporting.

26. The OECD workshop summary, including breakout group discussions, as well as major findings, recommendations and next steps are available in the document, Part 3: Workshop Report and Recommendations, in 2019 Workshop Report [ENV/JM/MONO(2020)35]. Table 2 below highlights the general recommendations to advance the development, application and acceptance of the AOP framework for use in MN risk assessment and decision making.

6 Conclusions from the project and recommendations

27. The OECD NanoAOP project contributes: (i) a systematic process for mining the nanotoxicity literature to identify potential KEs relevant for MNs and (ii) a strategy to prioritize identified potential KEs for future development. It demonstrates a methodology for developing KEs relevant for MNs, from evidence gathered from the literature. The methodology is shown in the tissue injury KE case study. In addition, the NanoAOP Database is a major contribution to this project. The recommendations in this report highlight key next steps to improve and keep this resource updated. The two workshops convened in 2018 and 2019, gathering a broad set of expertise provided a platform to (i) discuss approaches and methodologies developed in this project, and (ii) gather input on the status, use, and future needs for use of the AOPs in the risk assessment of MNs.

28. The NanoAOP project has established a methodology that can be applied to advance the future development of AOPs that will be useful for risk assessment and regulatory decision making about MNs. The methodology uses existing and available high quality nanotoxicity literature, mined and curated in a standardised format by experts, which is a cost-effective strategy to capture mechanisms and KEs important for MN risk assessments. The AOP framework can be used to systematically assess, use and interpret a large amount of alternative testing data for MNs that has been developed over the last decade. Based on the discussion, experts agreed that currently, the AOP framework can be used for: (i) ranking and prioritising MNs; (ii) identifying critical KEs to develop alternative testing strategies; (iii) product development as part of a safer manufacturing approach; and (iv) together with 'omics' strategies, AOPs can be used to propose testing that could be predictive of AOs. There is a need to identify which KEs are critical for testing as part of an IATA. Critical KEs could be prioritised by examining which events are shared across multiple AOPs. For each critical KE identified, AOPs can be used to help identify which methods/assays are appropriate and predictive for characterising them.

29. On the other side, a number of barriers toward using AOPs for MN risk assessment were also identified. For instance, there is a need to address the technical and translational issues of applying AOPs for MN risk assessment, including: limited quantitative and temporal data for KER and AOP development, a lack of guidance on the use of AOPs for quantitative risk assessment, and few accepted alternative testing methods to use as part of an IATA. A series of next steps are proposed to refine the methodologies developed, and to advance the adoption of AOPs for real-world applications in MN safety assessment and decision making, in the 2019 Workshop Report, Part 3: Workshop Report and Recommendations [ENV/JM/MONO(2020)35].

30. General recommendations include:

- ***Advance MN-relevant considerations in AOP development.*** The AOP framework requires considerations of the unique property associated changes in the biological mechanisms and thus, development of processes that allow identification of MN specific toxicity is needed. The methodology developed in the NanoAOP project can be applied to develop additional MN-relevant KEs.

- ***Utilize existing data.*** A database has been generated that will be useful for advancing the development, application and use of AOPs for MN risk assessment. In so far as possible, it is advantageous to mine, curate and use these data to advance knowledge and identify opportunities for additional AOP development. Significant additional efforts are required to evaluate the quality of available, published literature and bring these data together into searchable databases. The development of machine learning tools to automate literature searches; quality evaluations; and database development would be beneficial.
- ***Promote reliable and quantitative MN data development.*** Additional high quality, standardized and published data are required to ensure MN-relevant AOPs can be developed and used in decision making. Guidance is needed on the types of data and reporting standards to support their use in regulatory decision making. Efforts should be coordinated among stakeholders to ensure efficiency and limit additional animal testing.
- ***Identify current applications of the AOP framework for MN decision making.*** Select applications of the AOP framework have been proposed for current use in MN safety decision making. Guidance is recommended for use of AOPs in screening-level MN safety decisions that are fit-for-purpose (*e.g.* prioritization, grouping and categorisation).
- ***Establish test methods and protocols useful for MN decision making.*** Test methods to accurately measure MN-relevant MIEs and KEs are needed to advance use of AOPs in MN risk assessment. Development (and verification) of harmonized, standardized MN-relevant test methods linked to the selected KEs are needed.
- ***Demonstrate predictive capability of AOPs and in vitro test methods for MN decision making.*** To be useful for decision making, a coordinated effort is required to ensure the alternative testing models for KEs in an AOP are predictive of an adverse outcome of regulatory relevance. Development, testing and demonstration of the predictive capability of *in vitro* assays is required.
- ***Guidance to facilitate the adoption of MN-relevant AOPs for decision making.*** The science required to address the technical challenges of transitioning to alternative (non-animal) toxicity testing is progressing, but efforts are needed to translate and incorporate these developments into decision making about the safety of MNs. Guidance for risk assessors to analyze and evaluate AOPs for MN safety assessments is required and technical developments in alternative testing strategies need to be incorporated into regulatory guidance and policy documents.
- ***Stakeholder communication & engagement.*** To facilitate the development, adoption and use of the AOP framework for MN decision making, engagement of multiple stakeholders with a broad range of expertise is essential, and coordination as well as cooperation are needed. Future workshops targeted to encourage participation from various stakeholders with an interest in AOP development and application for MN decision making are critical.

31. Proposed next steps to advance and refine the methodologies developed in the NanoAOP project include:

- Update and refine the format, content, and layout of the NanoAOP database so it is optimized for KE development and analysis.
- Evaluate how different data sets (*e.g. in vitro* vs *in vivo* data) can factor into a weight of evidence analysis for supporting MN-relevant KE and AOP development.

- Critically review and incorporate additional information from other resources, including Registration, Evaluation, Authorisation, Restriction and Chemicals (REACH) dossiers, the NanoCommons project[15], and ToxCast.[16]
- Expand the methodology to address additional aspects of AOPs (*e.g.* KERs and MIEs)
- Expand the methodology to address additional considerations in MN safety assessments, such as structure-activity relationships between MN properties and biological outcomes.

[15] See https://www.nanocommons.eu/

[16] See https://www.epa.gov/chemical-research/toxcast-chemicals

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