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FOR NANOMATERIALS

Study on (bio)degradation, persistence and safe by design of nanomaterials

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List of abbreviations

2D - 2-Dimensional

3-Cltyr - 3-Chlorotyrosine

3-Notyr - 3-Nitrotyrosine

5-Ohmeu - 5-Hydroxymethyl Uracil

8-Ohdg - 8-Hydroxydeoxyguanosine

8-OHG - 8-Hydroxyguanosine

ALF - Artificial Lysosomal Fluid

ALI - Air–Liquid Interface

AFM - Atomic Force Microscopy

AOP - Adverse Outcome Pathways

APA - Aiegen ((9-Anthrylmethyl)Bis(2-Pyridylmethyl)Amine)

APMA - Aerosol Particle Mass Analyser

ASTM - American Society For Testing And Materials

ATR-IR - Attenuated Total Reflectance–IR

Baua - German Federal Institute For Occupational Safety And Health

CAGR - Compound Annual Growth Rate

CB – Control Banding

CEN - European Committee For Standardization

Cnts - Carbon Nanotubes

CSC - Cancer Stem Cells

CT - Cell Transformation

CTE - Column Transport Experiments

DAVID - Dosimetric Aerosol *In Vitro* Inhalation Device

Degs - Differentially Expressed Genes

DLS - Dynamic Light Scattering

DLVO - Derjaguin-Landau-Verwey-Overbeek

DOC - Dissolved Organic Content

EBC - Exhaled Breath Condensate

EC - European Commission

ECETOC - European Centre For Ecotoxicology And Toxicology Of Chemicals

ECHA - European Chemicals Agency

EDS - Energy-Dispersive X-Ray Spectroscopy

EELS - Electron Energy-Loss Spectroscopy

EFSA - European Food Safety Authority

ELPI - Electrical Low-Pressure Impactor

ELS - Extensive Literature Search

EPA - Environmental Protection Agency

EPR - Electron Paramagnetic Resonance

ESI-MS - Electrospray Ionization–Mass Spectrometry

ESR - Electron Spin Resonance

EU - European Union

EUON - European Union Observatory Of Nanomaterials

EURES - European Union System For The Evaluation Of Substances

EXAFS - Extended X-Ray Absorption Fine Structure

FFF - Flow Field-Flow Fractionation

FLG - Few Layer Graphene

FMPS - Fast Mobility Particle Sizer

FP7 - 7th Framework Program

FTIR - Fourier-Transform Infrared Spectroscopy

Gans - Generative Adversarial Networks

GC-MS - Gas Chromatography–Mass Spectrometry

GHS - Globally Harmonized System

GO - Graphene Oxide

Gpx - Glutathione Peroxidase

H2020 - Horizon 2020

Hbn - Hexagonal Boron Nitride

HES - Hazard Evaluation Strategies

HRP - Horseradish Peroxidase

HTS - High Throughput Screening

IC - Inorganic Carbon

ICP-AES - Inductively Coupled Plasma Absorption Emission Spectroscopy

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

ICP-OES - Inductively Coupled Plasma Optical Emission Spectroscopy

Ionps - Iron Oxide Nanoparticles

ISO - International Organization For Standardization

KE – Key Event

LCA - Lifecycle Analysis

Lcis - Life Cycle Inventories

Lip - Lignin Peroxidase

LCMS/MS - Liquid Chromatography–Mass Spectrometry

MAD - Mutual Acceptance Of Data

MCDA - Multi-Criteria Decision Analysis

MEMS - Micro-Electro-Mechanical Systems

MHW - Moderately Hard Reconstituted Water

Mies - Molecular Initiating Events

MMP-2 - Metalloproteinase-2

MNNG/HOS - Human Bone Osteosarcoma Cell Line

MOF - Metal Organic Frameworks

MPO - Myeloperoxidase Protein

MR - Magnetic Resonance

MWCNT – Multi-Walled Carbon Nanotube

NACIVT - Nano Aerosol Chamber For *In Vitro* Toxicity

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NET - Neutrophil Extracellular Trap

NIA - Nanotechnologies Industries Association

NIR - Near Infrared

NMs - Nanomaterials

NP - Nanoparticle

NR - Nanorod

NS - Nanosphere

NTA - Nanoparticle Tracking Analysis

O-Tyr - O-Tyrosine

OECD - Organisation For Economic Co-Operation And Development

OFC - Oxygen Flask Combustion

PAGE - Native Polyacrylamide Gel Electrophoresis

PCP - Poly-Cationic Peptide

PEG - Polyethylene Glycol

PEI - Polyethylenimine

PEO - Poly(Ethyleneoxide)

PFAS - Perfluoroalkyl And Polyfluoroalkyl Substances

PLGA - Poly(Lactic-Co-Glycolic) Acid

PMA - Phosphomolybdic Acid

PS - Pulmonary Surfactant

PUFP - Personal Ultrafine Particle Counter

QCM-D - Quartz Crystal Microbalance With Dissipation

QD - Quantum Dot

QRA - Quantitative Read-Across

QSAR - Quantitative Structure–Activity Relationship

R&D - Research And Development

RA - Risk Assessment

REACH - Registration, Evaluation, Authorisation And Restriction Of Chemicals

Rgoag - Reduced Graphene Oxide Ag Nanocomposite

ROS - Reactive Oxygen Species

RP - Regulatory Preparedness

Sapnets - Structure–Activity Prediction Networks

SAR - Structure-Activity Relationships

Sbd - Safe By Design

SEM - Scanning Electron Microscopy

SIA - Safe Innovation Approach

SME – Small And Medium Enterprises

SOD - Superoxide Dismutase

SPION - Superparamagnetic Iron Oxide Nanoparticles

SR - Systematic Review

SUNDS - SUN Decision Support System

SW - Synthetic Seawater

TEA - Triethanolamine

TEM - Transmission Electron Microscopy

TEOM - Tapered Element Oscillating Microbalance

TF - Task Force

TG - Testing Guidelines

TGA - Thermo-Gravimetric Analysis

TOC - Total Organic Carbon

Tof-SIMS - Time-Of-Flight Secondary Ion Mass Spectrometry

Usnps - Ultrasmall Nanoparticles

UV-Vis - Ultraviolet–Visible Spectroscopy

WBC - White Blood Cell

Woe - Weight-Of-Evidence

XAS - X-Ray Absorption Spectroscopy

XO - Xanthine Oxidase

XPS - X-Ray Photoelectron Spectroscopy

XRD - X-Ray Diffraction

Abstract

The current study was performed on behalf of the EU Observatory for Nanomaterials (EUON) and the European Chemicals Agency (ECHA). The study consisted of an extensive literature search (ELS) aimed at providing information on the state-of-the-art, the existing gaps, and the research needs of the degradation, biodegradation and persistence of nanomaterials and their relevant organic coatings. Another objective of the study was to examine the current state of the art of safety by design (SbD) for nanomaterials, as it relates to the degradation and persistence of nanomaterials. Here the aim was to test whether SbD of nanomaterials considers the (bio)degradation of nanomaterials and to provide recommendations to adapt existing SbD principles to consider the knowledge collected from literature on (bio)degradation of nanomaterials to reduce their persistence in the environment.

The study presents a literature-based update on the state-of-the-art of NMs and their organic coatings degradation and biodegradation, and the SbD of NMs. It covers a wide range of nanomaterials, i.e., metals, metal oxides, carbon-based NMs, and nanoliposomes. Information is provided on the techniques used to characterise the NMs and organic coatings (where applicable) and the study of (bio)degradation and SbD, as well as the existing gaps and recommendations on the future steps. Furthermore, an overview of existing test guidelines (e.g., OECD) are presented that are applicable, or can be applied following modifications for the study of NMs (bio)degradation and SbD. The literature search is complemented with respective surveys from relevant experts and the OECD.

1. Executive summary

1.1 Background

Upon the request of the European Chemicals Agency (ECHA) and the European Union Observatory of Nanomaterials (EUON) NovaMechanics Ltd. has undertaken a study on the (bio)degradation, persistence, and safe by design (SbD) for nanomaterials (NMs). The work performed is based on the NMs as defined in the EU adopted definition for the term nanomaterial in 2011 Recommendation on the definition of a nanomaterial (2011/696/EU).

The purpose of this report is to help ECHA/EUON to provide its stakeholders with reliable and transparent information on the safety and markets of NMs. As the number and complexity of NMs increases, there is a need to address how these nanomaterials will behave in the environment.

Thus, the key aim of this study was to address the degradation of NMs (including biodegradation where relevant, for either organic nanomaterials or organic coatings on nanomaterials). In addition, the study aims at examining what tools are available for the assessment of (bio)degradation, and how these can be used in different regulatory processes.

Another key aim of the study was to examine the current state of the art for SbD of NMs, as well, as it relates to the degradation and persistence of nanomaterials. The study examined how SbD of NMs considers the (bio)degradation of NMs and provides recommendations to adapt existing principles to consider the knowledge collected from literature on (bio)degradation of NMs to reduce their persistence in the environment.

1.2 Content of the study

As part of the on-going activities mandated to the EUON, the Observatory wished to conduct a study to assess the state of the art of NMs (bio)degradation, persistence, and SbD. The purpose of this study was:

- Collate state of the art information on tools and methods available to determine the (bio)degradation of NMs in a structured literature review;
- Collect and analyse if challenges have been identified on the methods and what are the identified gaps in (bio)degradation test methods and results for NMs;
- Collate state of the art information on SbD strategies for NMs to reduce environmental risks;
- Complement the structured literature reviews with surveys of experts leading in the field;
- Conclude on current approaches and gaps to assess persistency and (bio)degradation of NMs in the environment as well as current SbD strategies.

1.3 Methodology

The report was compiled following a detailed step-by-step strategy to gather the required data and provide EUON and the EUON stakeholders with the necessary insights. To achieve this, the study was divided into subtasks:

1. The structured literature review: The first subtask performed the literature review on two main topics: a) persistency and (bio)degradation of NMs and available tools for their assessment, and b) safe-by-design approaches for NMs to enhance the development of environmentally benign materials.

2. Expert identification: In parallel with subtask 1, subtask 2 identified leading experts and sought their opinion regarding the (bio)degradation of NMs and organic coatings as well as SbD strategies for reducing the risks of environmental exposure.

3. Survey/interviews with identified experts: Based on the results of subtasks 1 and 2, two surveys were designed, refined in collaboration with ECHA and conducted with identified experts to collect information on (bio)degradation of NMs as well as SbD for reducing the risks of environmental exposure and hazard.

4. Summary and results presentation: Based on all acquired data from subtasks 1-4, a report was produced with conclusions reached, emphasising the gap identification and outlook to drive future developments in the areas of (bio)degradation of NMs and SbD strategies.

1.3.1 Literature search methodology

The methodology followed to perform the secondary (literature) research (see section 3.1) of publicly available data was based on standardised processes for performing a robust Systematic Review (SR) based on European Food Safety Authority (EFSA) and other EU institutions guidelines. These steps include:

1. Identify, select, and critically evaluate relevant research and data sources

2. Evaluate and synthesise the current body of knowledge (on (bio)degradation of NMs and organic coatings and the SbD of NMs).

This methodology ensured that:

1. The study was based on sound scientific pillars to enhance the reproducibility of the study.

2. Increase the credibility and transparency of the outputs, so that they can be considered as the most relevant findings from scientific literature and industry reports in the field.

3. Provide to ECHA/EUON and its stakeholders timely information on the state of the art of the required topics.

4. Analyse, explore, and present the results in a systematic, comprehensive, and informative way.

1.3.2 ELS on the (bio)degradation of NMs and organic coatings and SbD of NMs

To identify relevant high-quality literature, extract the required information, and information on involved leading experts the following steps took place:

1. A detailed search protocol was developed and implemented, based on the tools developed by NovaMechanics Ltd. for literature search and retrieval.

2. Collection and extraction of information from literature based on predefined set of key criteria.

3. Identification of leading experts.

4. Formulate questionnaires that were used for retrieving further information regarding the NMs and organic coatings (bio)degradation and NMs SbD.

The basis of the presented research was the current EC definition of NMs, which dates from 2011. This allowed to create a baseline that was used for the identification and refinement of

the current scientific landscape. This was achieved through the search, mining, and data extraction from high-quality peer-reviewed literature, and official policy and other reports, as well as web research in relevant databases (e.g., EUON, PubChem, ChEMBL, Google, Google Scholar, PubMed, Scopus, SciVal, the Publications Office of the EU). Any of the identified literature was evaluated on their quality (see section 3.1) and those meeting the required threshold were used for analysis.

1.3.3 Identification of leading experts

Based on the results obtained from the literature search, ongoing research projects, Novamechanics participation in relevant associations, and its scientific and commercial network made it possible to identify leading experts in the fields of NMs and organic coatings (bio)degradation and NMs SbD.

1.3.4 Surveys with identified experts

Following collection, extraction, and analysis of data from the literature search, Novamechanics developed in collaboration with ECHA/EUON targeted questionnaires on the topics of NMs (bio)degradation and organic coatings and SbD of NMs. These were shared with the identified experts and the responses were collected, analysed, and compared with the results of the literature search.

On a second instance, OECD relevant experts were contacted to gather relevant views, state-of-the-art, and ongoing actions at the OECD level.

1.3.5 Data synthesis, analysis, and reporting

The last part of the study was to gather and synthesise the information gathered from all sources, analyse the results, and produce a final report that includes the results and with conclusions reached, emphasising the gap identification and outlook to drive future developments in the areas of (bio)degradation of NMs and SbD strategies.

1.4 Findings

This report presents the findings of an extended literature study and surveys of leading experts in the fields of NMs and organic coatings (bio)degradation and NMs SbD. The report covers a wide range of NMs including:

- Metal oxides
- Metals
- Carbon-based NMs
- Nanoliposomes

The key findings of the study are:

- NMs and organic coatings (bio)degradation:
    - (Bio)degradation and persistence studies focus mainly on organic nanomaterials (NMs) and/or organic coatings on nanomaterials.
    - Enzymatic degradation is the most widely used method for studying the degradation of NMs. Enzymatic degradation of NMs can lead to changes of their physicochemical and structural properties (e.g., through surface adsorption

forming a corona). Furthermore, the NMs – enzyme interaction can lead to the inhibition of enhancement of enzyme activity (e.g., blocking the enzyme active sites) with favourable or unfavourable results.

- Cell and bacterial degradation have also been employed. Cell degradation (e.g., macrophages) can be initiated using organic coatings in NMs (e.g., phosphatidylserine) that act as a trigger for carbon-based NMs digestion by cells.

- A wide range of experimental and computational techniques have been used for the monitoring, studying and quantification of (bio)degradation and persistence. These include imaging (e.g., electron microscopy), spectroscopy (e.g., Raman), labelling (e.g., ^{14}C), tracking (e.g., nanoparticle tracking analysis) and other techniques. The detection limits and resolution of these techniques, though, needs to be enhanced to study the (bio)degradation of NMs and their organic coatings in detail.

- Novel techniques, such as synchrotron-based X-ray microscopic and EXAFS (Extended X-ray absorption fine structure) analysis, can substantially help with the study of (bio)degradation and persistence in complex environments.

- Substantial work is still needed for the study of NMs degradation and persistence in complex environments (e.g., homoaggregation, homoagglomeration).

- Several techniques used for the study of conventional chemicals degradation and persistence are also being applied for NMs.

- Most of the test guidelines used for studying the degradation and persistence of chemicals have been applied to NMs with or without modifications.

- In terms of environmental fate – and therefore in a broader sense related to persistency - there is currently only one standardised test guideline available (OECD TG 318) looking into 'Dispersion stability of Nanomaterials in Simulated Environmental Media', i.e. assessing the ability of a nanomaterial to attain a colloidal dispersion and to conserve this dispersion under environmentally relevant conditions.

- No predictive modelling studies exist that focus on the degradation or persistence of NMs.

- SbD of NMs:

  - There have been many efforts for establishing a definition or a clear concept for SbD of NMs, but it still seems challenging to establish consensus for a unique definition for the SbD of NMs.

  - Two main reasons for this challenge:

    - The vagueness and broadness of the term "safety".

    - The unique and different properties of NMs compared to bulk chemicals, which may require a case-by-case safety or risk assessment.

  - An active and vibrant topic of research with many "Safe by design", and perhaps even more "Safer by design" strategies for NMs being developed. Care needs to be taken whether the terms Safe and Safer in fact means the same.

  - There are already proposed SbD strategies available for NMs, for which the efforts

and push for their improvement and implementation are ongoing.

- The boundaries regarding the SbD of NMs include their unique nature, differences in behaviour for the same NM in different environments, the lack of sufficient high-quality data and tools, and the lack of consensus on the safe development and handling of nanomaterials among the various stakeholder groups.
- In most cases, existing SbD strategies for conventional chemicals and other substances require modifications to be applied to NMs.
- Emerging approaches for SbD include mainly computational workflows, development of customised NMs with specific properties, and development of specialised experimental techniques.
- Transparency, public communication, and demonstration of the positive effects of NMs are key for societal acceptance.

- Summary of the responses to the expert surveys:
  - The responses received covered a wide range of nanomaterials (NMs) for both (bio)degradation and safe by design (SbD).
  - For NMs (bio)degradation relevant agreement existed on the definition. This focussed on the physical or chemical transformation under different relevant conditions.
  - The proposed definitions were also applicable to organic coatings of nanomaterials.
  - The techniques mentioned to study/monitor the (bio)degradation of NMs and organic coatings agreed with those identified during the literature search.
  - Some existing guidelines were mentioned (mainly OECD) but fell short from those identified during the literature search.
  - Lack of systematic and scientific data was mentioned as a gap for both (bio)degradation and SbD.
  - For (bio)degradation targeted evaluation of existing methods should take place. Combination of methods will be required for a complete strategy for NMs and organic coatings, while improving qualitative as well as quantitative detection limits where possible.
  - Research should also focus on the by-products of the (bio)degradation, their long-term effects, and their fate.
  - A common definition for SbD could be possible, but most likely would have to be sector specific.
  - The SbD definition should cover the prediction and identification of adverse NMs effects and working on reducing this risk.
  - There is some NMs SbD implementation at an industrial scale, but more work is needed.
  - There is a wide range of ongoing actions and strategy development for NMs.

- Boundaries regarding the implementation of SbD strategies in real-life scenarios is hindered by the lack of scientific data, the testing in non-real-life cases, and the loss of NM functionality.
- Sbd should be part of but should not replace regulatory evaluation.
- It was suggested by 30% of the survey participants that the sustainability domain should be part of the SbD framework and specifically integrate the environmental and societal dimension.
- NMs will need to be re-evaluated if modified strategies are implemented, but this will most probably be sector specific.
- Research supported by public funding should focus on developing standardised testing approaches to thoroughly risk assess SbD nanomaterials
- Public communication of SbD strategies can help with societal acceptance of NMs-containing products.

2. Introduction

The main aim of this project is to provide a comprehensive study on the persistence, (bio)degradation and safe by design (SbD) of nanomaterials (NMs). The study requires an optimised combination of primary and secondary research to be successful. Secondary research, in the form of literature mining, will take place with the purpose to identify knowledge gaps in the case of NMs. This includes the fate and degradation of NMs following exposure (including biodegradation where relevant, for either organic nanomaterials or organic coatings on nanomaterials) and whether methods exist to assess these or if it is possible to apply (with specific modifications if necessary) methods from other substances to NMs, focussing on abiotic processes. Similarly, the same approach will be followed regarding SbD strategies for NMs, including whether these strategies can assist with developing environmentally benign NMs and promote societal acceptance of NMs-containing products. The literature search will be based on respective materials from peer-reviewed scientific literature, literature reviews on the topic if available, publicly available reports from research projects, agencies and regulatory bodies mined using the state-of-the-art Enalos+ tools developed by NovaMechanics Ltd. for automated literature mining and exploitation.

The literature search will help to identify gaps and challenges in the assessment of the (bio)degradation of NMs and their organic coatings and in the SbD of NMs. This will include any required changes to make existing SbD strategies applicable to NMs. The results from the literature search will be complemented with primary research, which will include direct communication with relevant stakeholders (i.e., manufacturers, regulators, researchers) in the form of questionnaires, face-to-face and online interviews and focus groups. The resulting goal is to structure, harmonise and combine the results from both the primary and secondary research and thereby to provide a robust and validated analysis on the suitability of existing tools and methods to analyse persistence and (bio)degradation of NMs and the potential transfer into application in a regulatory context while highlighting gaps and development needs. In this context, the relevance and applicability of emerging "functional assays" will also be considered. Furthermore, the research will provide insights into the developmental needs in application of the scientific principle of SbD to meet NM-specific challenges and whether SbD concepts for NMs will improve the overall governance and societal acceptance of NMs-containing products.

The project will utilise the EU adopted definition for the term NM as per the 2011 Recommendation on the definition of a nanomaterial (2011/696/EU) [1]. NMs have become part of everyday life as they are being used extensively in a wide range of consumer goods (e.g., cosmetics, food additives) and industrial products such as solar cells and paints. Based on

previous studies [2], [3] the European NMs market was estimated to grow with a Compound Annual Growth Rate (CAGR) of 20% for the period of 2016 - 2022, with a later study [4] projecting the NMs market to register a CAGR of 15.24% in terms of revenue during the period 2020-2028. This, combined with the emergence of novel complex and advanced materials, leads to increased environmental release and exposure risks. Hence, understanding the environmental behaviour of NMs is key, when it comes to environmental risk and hazard assessment, including the persistence and (bio)degradation of organic and inorganic NMs and their organic coatings and stabilisers.

NMs (bio)degradation can have a substantial effect on their hazardous potential, irrespective of which the main NMs degradation mechanisms undergoes, i.e., chemical degradation or biodegradation [5], [6]. In the case of nanomedicines biodegradation can be beneficial (i.e., targeting specific biological sites and controlled medicine release) [6]. Thus studying, decoding, and eventually controlling the (bio)degradation of NMs can have beneficial effects. To achieve this, SbD strategies are required that will allow control over the behaviour of NMs in complex environmental and biological media to take advantage of their unique properties, maximise their beneficial potential, while decreasing their hazardous potential for human health and the environment.

To achieve this, SbD strategies are being developed considering both exposure and hazard aspects [7]–[9], while the EU has funded several projects (i.e., ASINA, SaByNa, SABYDOMA, SUNSHINE, DIAGONAL, HARMLESS, SbD4Nano, CompSafeNano - coordinated by the contractor) to provide a SbD framework for NMs. To meet these goals, a dual approach is required. This includes R&D practices at an experimental and computational level and in parallel detailed and continuous communication between regulators, industry and academia. As a result, specific actions and guidelines are needed to complement experimental data regarding NMs fate and behaviour to promote and achieve the key pillars of SbD of decreased hazardous potential, sustainable functionality, benefit to circular economy and key stakeholder engagement.

3. Methodology

3.1 Extensive Literature Search

The methodology included the development of a protocol with standardised processes for performing a robust Extensive Literature Search (ELS) in combination with the principles of a Systematic Review (SR), as endorsed by EFSA and other EU institutions [10], [11] and customised to match the project's objectives. This covers all steps including identifying, selecting, and critically appraising relevant research and data sources, appraising, and synthesising the current body of knowledge on the (bio)degradation and persistence of NMs and existing relevant strategies. Structuring the methodology based on the principles of SR and ELS, ensured: i) that the entire study was based on sound scientific pillars, which will enhance the reproducibility of the study, ii) increased credibility and transparency of the outputs of the ELS, so that they can be considered as the most relevant findings from the relevant literature in the field, iii) that EUON/ECHA acquire access to timely information on the state of the art, and iv) that identified results are analysed, explored and presented in a systematic, comprehensive and informative way, which is useful and interpretable by EUON/ECHA and other stakeholders.

During the protocol development, the review questions and scope, the methods of the SR, and the eligibility criteria for the inclusion of studies/reports and materials into the current ELS were defined. This reduced bias, and as the process is clearly specified, it was possible for ECHA to comment and suggest revisions, while the reviewing team followed and customised the documented process as needed and based on relevant feedback. In addition, the extensiveness and reproducibility of the search strategy and the transparent reporting of how studies are selected and included in the ELS also reduced bias in the selection of research studies. The search strategy is reported below to allow readers to judge how much of the relevant literature

is likely to have been found.

Furthermore, the ELS allows assessment of the quality of the evidence in terms of study methodological soundness, which gives an indication of the strength of evidence provided by the review and allows emphasis to be given to the results from studies/sources of higher quality. This allows readers to critically appraise the judgments made in the study selection and the collection, analysis, and interpretation of the results.

Overall, the ELS follows closely the fundamental principles of systematic reviews, i.e., i) methodological rigour and coherence in the retrieval and selection of studies/sources, assessment of their methodological quality, and the synthesis and interpretation of information, ii) transparency, and iii) reproducibility, while the ELS characteristics have been adjusted to comply with SR characteristics where necessary, as shown in Table 1.

Table 1: Characteristics and description of aligned ELS and SR

#	Characteristics	Description
1	Study questions	Focused and explicit
2	Eligibility criteria for inclusion or exclusion of studies	Pre-defined and documented; objectively applied
3	Description of the review method	Reported and predefined in a protocol
4	Literature search	Structured to identify as many relevant studies as possible
5	Methodological quality assessment of included studies	Included, typically using a quality assessment tool
6	Reporting of study results	Full reporting of relevant results (numerical results)
7	Synthesis	Quantitative synthesis (meta-analysis) when possible

General method for a systematic review

The core steps of a systematic review are illustrated in Figure 1. Each step must be carefully documented in the SR to ensure transparency and reproducibility.

Based on the sources identified text mining and manual data extraction and information techniques and tools were used to maximize the information retrieved.

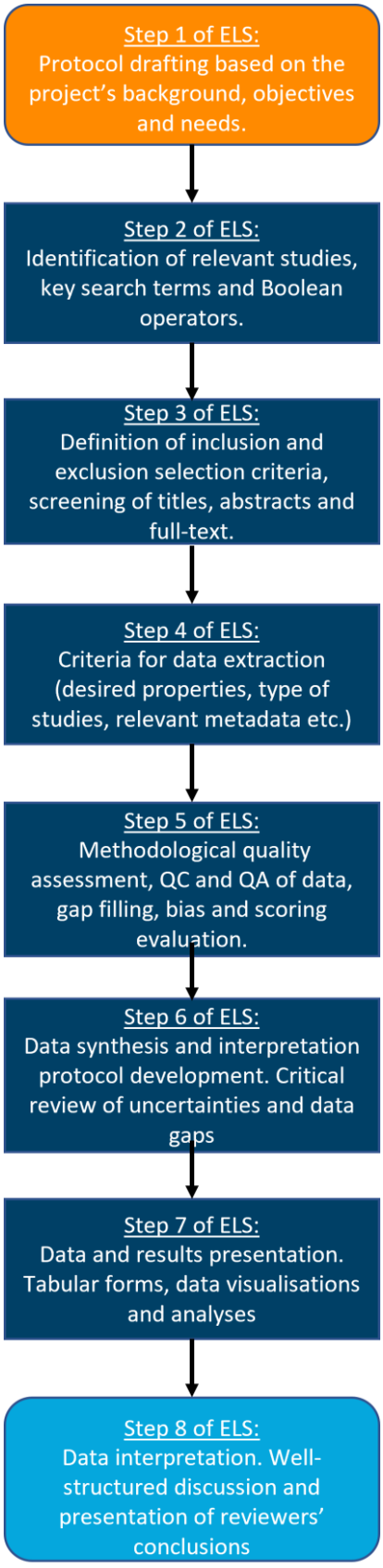

Figure 1: Core steps for performing a systematic review (as adapted by [10]), from the Cochrane Handbook for Systematic Reviews of Interventions, [11]).

Search strategy protocol

The development of the search strategy protocol was based on the project's main objective, which is to provide the EUON stakeholders with insight into the degradation of NMs (including biodegradation where relevant, for either organic NMs or organic coatings on NMs) and examining what tools are available for the assessment of (bio)degradation, and how these can be used in different regulatory processes. Based on the key aims a set of key questions, regarding the (bio)degradation and persistence of NMs, have been identified that will act as the basis for the keywords that need to be addressed through the ELS. The key questions include:

a. What are the tools and methods used to determine NMs persistence and biodegradation?
b. Do these methods, especially standardised ones, match those for other substances?
c. Are there methods specifically applicable to organic NMs and coatings?
d. Are there methods able to assess degradation focussing on abiotic processes?
e. What are the challenges when assessing NMs persistence and biodegradation?
f. Are there promising Functional Assays[1] available or under development that could be useful for regulatory purposes?
g. What are the gaps regarding the (bio)degradation test methods and (bio)degradation results of NMs?

Definition of search terms and Boolean operators

Based on key questions defined in the previous section, the keywords used for retrieving the relevant literature were defined. The search included a series of general searches, nanomaterial AND (degradation OR persistence). Further refinement included key terms based on the project's objectives, i.e., the existence of specific strategies, characterisation methods, NMs fate, NMs persistence, regulatory approaches etc. Literature research was also performed using key terms that were identified based on the gaps and challenges identified, to acquire relevant literature that may have not been retrieved during the main search process.

To maximise high quality data retrieval, a balance between the specificity and sensitivity of the search terms was established. These two approaches are key to an effective search strategy, with both presenting relevant advantages and disadvantages [13]. A specific search provides a substantial amount of relevant research, while avoiding, to a large degree, irrelevant results. In this way there is a substantial amount of time saving when it comes to filtering and screening the results. The disadvantage is that the more specific the search becomes, the higher the risk of missing relevant literature. This is because specific search relies on searching very precise concepts and ideas and their combinations, while focussing on specific parts of the study, e.g., title and abstract. This can lead to data loss in the case that non-standard, modified or novel terminology is used, or where a vague title and/or generalised abstract are present [13], [14].

On the other hand, sensitivity provides researchers with the opportunity to capture the majority of relevant literature and substantially lowers the risk of relevant data loss [13]. The disadvantage, in this case, is the fact that more irrelevant literature is retrieved as well as the useful literature. As a result, the time and effort required for filtering and screening increases. This is because a sensitivity-focussed search relies on using more generalised terms and their combination and does not focus on specific study parts. This will lead to more hits, both relevant and irrelevant, that will need to be carefully screened to discard unwanted studies [13], [14].

Summarising, a specific search is aimed at answering research questions with a high degree of

[1]Functional Assays are intermediary, semi-empirical measures of processes or functions within a specified system that bridge the gap between NM properties and potential outcomes in complex systems. The three components of a functional assay are standardized protocols for parameter determination and reporting, a theoretical context for parameter application and reference systems. See Hendren et al. for further details [12].

certainty, using clearly defined and specific search terms. Sensitivity is required when the researchers are looking to perform an exhaustive literature search and when the concepts and the questions needed to be addressed are not clearly defined. In this case, the optimum balance between the two concepts was required, while not restricting the search to one or limited databases or specific parts of literature [15], [16].

Resources' selection criteria

As per the technical specification requirements, a clear set of resource inclusion and exclusion criteria was defined in collaboration with ECHA to ensure high quality outcomes. The inclusion and exclusion criteria are mainly based on the key questions presented previously and the project's requirements and complemented with general search criteria. The inclusion and exclusion criteria proposed (based on applicability) for the (bio)degradation and persistence of NMs are:

- Criteria for resource inclusion:
  - English Language publications.
  - Resources post 2010.
  - Resources discussing standardised and authorised persistence and (bio)degradation strategies.
  - Resources dealing with tools, methods or assays used to determine NMs (and polymer coatings) persistence and biodegradation.
  - Resources describing the applicability of standardised methods of other substances regarding persistence and biodegradation.
  - Resources describing the applicability or the potential applicability of substances methods to NMs. Persistence and biodegradation methods specifically applicable to organic NMs and coatings.
  - Methods able to assess degradation focussing on abiotic processes.
  - Resources discussing the challenges when assessing NMs persistence and biodegradation.
  - Resources discussing the gaps regarding the (bio)degradation test methods and results of NMs.

- Criteria for resource exclusion:
  - Resources not in English.
  - Resources prior to 2010.
  - Resources in predatory journals (even if peer-reviewed).
  - Non-peer-reviewed resources without references.
  - Resources without full text access.
  - Resources not describing the methodology used and required metadata in full (research studies).

Criteria for resource and methodological quality assessment

The identified studies were initially reviewed for compliance with the inclusion/exclusion criteria based on titles/abstracts, as described above, and then assessed based on the full-text content. For a resource to qualify for data extraction a minimum set of criteria needed to be met, which was defined in cooperation with ECHA. The evaluation criteria were used for resource and methodological quality assessment, in terms of data and metadata (methodological) quality and completeness. The criteria identified for resource and methodological quality assessment included:

- Information on types of NMs covered;
- Information on number of NMs covered;
- Information on which topics are covered: (bio)degradation and persistence strategies;
- Information on environmental compartments covered;
- Information on some or all of the following NMs characteristics: core material/structure, coatings, stabilizers, size, shape, susceptibility to ion or ligand release, potential hazardous residues from the synthesis;

- Information on tools and methods available to determine the (bio)degradation and persistence of NMs;
- Sufficient information must be provided to enable evaluation of the validity and suitability of the selected test methods and test guidelines;

3.2 Surveys with relevant experts

The objective of WP4 was to contact the identified experts on the topic of (bio)degradation of NMs as well as safe by design strategies for reducing the risks of environmental exposure, based on the findings and data acquired from the literature reviews of WP1-2 and ask for their expert opinion on both topics.

The research team designed two questionnaires, each one focussing on a single topic, i.e., (bio)degradation of NMs and organic coatings and SbD of NMs. The identified experts were divided in 3 categories:

i. (Bio)degradation experts

ii. SbD experts

iii. Experts covering both fields

Besides the experts identified though the literature search, Novamechanics used its well-established network and project participation to share the questionnaires, which included experts with strong background on both chemicals and NMs assessment, fate and SbD. The research team has established contacts and collaborations with multiple stakeholders through its participation in the EU NanoSafetyCluster, NIA, BioNanoNet and various H2020 research projects, including on NMs fate, risk governance of NMs, development of tailored nanoinformatics tools, Safety-by-Design and Safe Innovation Approaches.

Through this exercise, representatives from the following stakeholders have been contacted:

- Industry
- Industry associations (e.g., NIA, BioNanoNet)
- The OECD
- The EU NanoSafety Cluster
- Researchers (private and academia)
- Regulatory consultants

3.3 Methodological approach for (bio)degradation and SbD surveys

The aim of the surveys was to answer a set of key questions:

i. Research progress on suitability of tools and methods to analyse persistency and (bio)degradation of NMs and the potential transfer into applicability in a regulatory context while highlighting gaps and development needs;

ii. further development needs of the scientific principles of SbD to meet NM specific challenges;

iii. if and how SbD concepts for NMs will improve the overall governance and societal acceptance of NMs;

3.3.1 Organisation, preparation, distribution of the questionnaires and collection of the data

The questionnaires (see Annex for the actual questionnaires and included questions) were prepared based on the results of the ELS study from WPs 1 and 2. As stated previously, two questionnaires were prepared to cover key areas of both topics. As a result, five sections were added in each questionnaire. For (bio)degradation these were:

- Section 1. Personal information
- Section 2. Definition of (bio)degradation
- Section 3. (Bio)degradation strategies and techniques
- Section 4. (Bio)degradation and regulation
- Section 5. (Bio)degradation gaps and future steps

For SbD the sections were:

- Section 1. Personal information
- Section 2. Definition of safe by design (SbD) of nanomaterials
- Section 3. SbD strategies and techniques
- Section 4. SbD and regulation
- Section 5. SbD gaps and future steps

The questionnaires were distributed to the target audience, as identified in the stakeholder database.

The process developed for sharing the questionnaires, aimed at maximising the impact of the survey and ensuring that any fatigue in filing these is avoided. This process included:

- Set clear, attainable survey goals that were identified from the data gathered in WPs 1 and 2;
- Define a clear set of questions per topic to cover the required outcomes;
- Use the proposed questions to draft more analytical questions that were customised for different topics ((bio)degradation vs. SbD) and on the purpose of data they are focused on in collaboration with ECHA;
- Pick the best questions that will lead to data retrieval maximisation at the optimum time frame (max 20 minutes for online questionnaires, and preferably no more than 15 minutes);
- Craft questions to maximise the acquired results, with an optimal ratio of closed (e.g., yes/no, multiple choice, agreement/disagreement) to open-ended (e.g., free-text, critical, personal opinion) questions;
- Ordering the questions so that the most significant questions are answered first, as there

is a chance that people start, but do not finish the questionnaire (to facilitate this a "submit now" button will be available throughout) ensuring all answers are captured.

Great care was taken to ensure that the questions were as short and as simply framed as possible. Similarly, the questions were reviewed to ensure that no bias is introduced in any way that may influence the participants answers. An example of biased and unbiased questions under the same context is:

- Biased: Do you think that the current regulatory framework helps with the development of SbD strategies for NMs?

- Unbiased: What are the barriers, if any, in your opinion, for the development of SbD strategies for NMs?

In all cases, participants were clearly informed regarding the scope and desired outcomes from the questionnaire and ensured anonymity by including an introductory letter to each presenting these details. The handling of confidential information was considered as well, as participants were able to flag any information/question as such for each section.

These participants were contacted via personal email and:

- All experts were given the opportunity to fill in the questionnaire online, via the NovaMechanics website, using a secure environment, fill in a Word version of the questionnaire offline or answer the questions via a live remote interview.

- All experts were informed that the questionnaires would be shared with ECHA in confidentiality and all published responses will be anonymised removing any personal or commercial data.

- There was the provision for questions to be flagged as confidential. In this case, the responses and data would be separated from the non-confidential data and shared with ECHA in the annex of the report but would not be mentioned or published in any way. They would be used though for reaching conclusions and any numerical or statistical analysis.

The questionnaires were put together in close collaboration with ECHA/EUON and based on current market research best practices [17], questionnaire duration did not exceed 20 minutes, with an optimum duration of around 15 minutes. The questionnaires were divided into sections, as presented above, with each section addressing a single topic to keep the mind of the participant focussed on all times. Where possible, questions did not require free text import to reduce the time and effort needed from the participants. These questions included multiple choice answers, agreement/disagreement degree etc.

3.3.2 Risk mitigation measures

The involvement of external stakeholders inherently imposes uncertainty for conducting surveys/interviews and the collection of the relevant data, as the collaboration and timely participation is difficult to ensure.

Timing of activities. The time plan was organised efficiently, taking advantage of project management experience and tools for timely implementation and delivery of the expected results. To minimise the impacts and ensure low possibility of risk appearances during the implementation of the tender, the management team took precautionary measures in designing the study, to mitigate against risks in relation to limited stakeholder participation as follows:

- Expert engagement. The scientific team is lead and composed of internationally recognised professional in their fields with existing strong networks of contacts who are

likely to engage;

- Increase awareness. The scientific team has vast experience on stakeholder consultation and contact with industry, regulators, and professional organisations to build project awareness and buzz.

- Data mining and handling. The scientific team has access to state-of-the-art IT tools that can be used for automatic mining, extraction and handling of data from multiple sources including websites;

- Project management. The scientific team is composed of experts that have coordinated large multi-sector and multi-stakeholder EU research projects.

Specific risk mitigation measures and actions have been identified and have been applied to ensure smooth implementation of WP4 activities.

Table 2. Identified risks and risk mitigation measures for the surveys.

Identified risks	**Severity / Possibility**	**Mitigation measures**
Low participation hinders the richness of the obtained data	High / Medium	*Preventive*: Establish contacts immediately upon contract award, enrich stakeholder database, establish good practices for communication, raise trust and stakeholder engagement. *Corrective*: Use existing networks to identify alternative contact persons. Offer easy formats for collaboration.
Stakeholder groups are unbalanced (e.g., absence of representative samples of a group, over-representation of samples of a group)	Medium / Low	*Preventive*: Equal number of members of stakeholder groups will be contacted and invited for participation. *Corrective*: The organisation of the stakeholder database will allow early identification of under/over-representation so that stakeholder groups can be balanced by inviting extra members or by adjusting the weighting of larger groups.
Delays in receiving replies	High / Medium	*Preventive-Corrective*: Include a survey incentive for responses within a particular time limit (e.g., a donation to Unicef's donate a vaccine for Covid-19 programme). Incentives are a great way to not only to increase response rates, but to also thank respondents for their time.
Length of survey / Incomplete surveys submission	High / Medium	*Preventive*: Engagement of stakeholders can be heavily influenced by the time needed to complete the surveys. The design of the questionnaire in WP2 will ensure that the survey will not require more than 30 min to be completed. *Corrective*: Information will be confirmed with stakeholders during the initial tasks in WP1 and the survey length can be reduced if deemed necessary for maximising participation.

4. Results

4.1 Extensive literature search on the biodegradation of nanomaterials and organic coatings

The ELS provided an extensive number of studies with respect the (bio)degradation and persistence of NMs and dealt with both human health (*in vivo* and *in vitro*) experiments and with their environmental fate. Out of all the papers screened, the most significant and of highest quality are reported here. Furthermore, for each presented section an iterative screening approach was used concluding this part of the work only when further study analyses did not bring any new information but repeated instead content previously extracted.

4.1.1 Carbon-based NMs

The results retrieved cover different forms of NMs like carbon-based (e.g., carbon nanotubes, graphene), metals, metal oxides and more. In general, NMs' (bio)degradation and persistence is a topic, which is widely studied due to their potential hazardous effects [18]. Reviews by Chen et al. [18], Sureshbabu et al. [19] and Modugno et al. [20] suggested that the enzymatic (bio)degradation of carbon-based NMs should be assessed, due to their increased use and potential biomedical applications and the hazardous potential of the degradation products when compared to the intact NM.

In the case of carbon-based NMs extensive research has been performed with respect to their microbial, cell and enzymatic degradation [18], [21].

- The microbes used for studying the degradation of NMs were naphthalene-degrading bacteria such as Pseudomonas [22], *Trabusiella guamensis* [23] and *Burkholderia kururiensis*, *Delftia acidovorans*, and *Stenotrophomonas maltophilia* [24] bacteria and *Sparassis latifolia* [25], *Phanerochaete chrysosporium* [26], [27] and chlorobacterium [28], *Trametes versicolor* and natural microbial cultures fungi [29].
- Cell degradation has been studied using intracellular macrophage degradation [30]–[32] and human blood plasma [33].
- In the case of the enzymatic degradation the enzymes that have been found to degrade carbon-based NMs were Lactoperoxidase [34], [35], Horseradish peroxidase (HRP) [19], [20], [35]–[39], Myeloperoxidase [32], [40]–[46], Xanthine oxidase [19], Eosinophil peroxidase [47], Lignin peroxidase (LiP) [25], [26], MnP [27], while Tyrosinase [24] and Laccase [24], [27] were not able to do so.

Furthermore, due to their distinct physicochemical properties, carbon-based NMs have been studied with respect their usefulness on controlling environmental pollution, their fate and degradation [5], which can be divided into photodegradation and chemical degradation [5], [48], [49], with the former relying on the interaction of NMs with light (mainly ultraviolet [48], [50]) and the latter on interaction with existing chemical reagents [5]. In the case of photodegradation, the addition of chemicals can substantially increase the degradation rate [49], which is more environmentally relevant since the reactions of NMs in the environment will be affected by other available substances [5]. Chemical degradation is based on the presence of strong oxidising agents like sulphide, O_3 and H_2O_2 [49], [51], while care needs to be taken during chemical degradation as in some reducing agents (e.g., chlorination) may lead to secondary pollution [52]. The effect of such agents have been tested using different methodologies like oxygen flask combustion [50] and column transport experiments [51]. A recent review paper highlighted a gap in existing studies and current understanding of the role of the acquired biomolecule corona around carbon-based NMs on their biodegradation [53].

4.1.2 Organic NMs and organic coatings

In general, it has been demonstrated that "soft" nanostructures (such as biopolymers, like lipids) are more easily degraded than "hard" nanostructures, such as metal (e.g., Au, Ag, etc.), metal oxide (e.g., ZnO, TiO_2, etc.), and carbon-based nanomaterials (e.g., CNTs, graphene, fullerene, etc.), which are typically more persistent *in vivo* [9]. As a result, modification of the surface of NMs using organic functional groups can assist with enhancing degradation and tune bio-persistence. For example, Seré et al. [54] demonstrated that mesoporous silica NMs functionalized with triethanolamine (TEA) degraded much faster than both carboxylated and nonfunctionalized ones. It was suggested that increased density of functional groups and defectiveness of carbon NMs may allow closer proximity with the active site of enzymes, and therefore facilitate faster degradation. Similarly, the persistence and lack of biodegradability of Nisin-Loaded Chitosan/Alginate NMs on the growth of *Staphylococcus aureus* in raw and pasteurized milk samples [55] and their use for encapsulation and release of vitamin B_2 [56] has been demonstrated.

In nanomedicine, the degradation of P(AAm-co-MAA) nanogels following the introduction of a biodegradable cross-linker has been studied using a quartz crystal microbalance with dissipation (QCM-D) and Dynamic Light Scattering (DLS) [57]. Illes et al. studied the degradation of exosome-treated iron-based (iron and fumaric acid) metal organic frameworks (MOF) and the controlled degradation of the exosome coating under biologically relevant conditions using Triton X-100 and artificial lysosomal fluid (ALF), with Triton X-100 leading to the degradation of the exosome coating and ALF decomposing both the coating and MOF [58].

The degradation of the organic (polymeric) coating has also been studied using poly(ethyleneoxide) PEO brush NMs (cross-linked bottle brush copolymers) and the phosphomolybdic acid (PMA) assay according to the Stevenson method, DLS and TEM [59]. The topic has been extensively studied in the case of nanomedicine, where organic coatings are used to stabilise the NM and control drug release and/or penetration into specific tissues and cellular uptake. Several studies have studied the biodegradation of either PLGA NMs [60]–[62] and PLGA-coated iron oxide NMs [63], [64] following cellular uptake, inducing autophagy and being degraded via an autophagy-lysosome pathway.

4.1.3 Metallic and other NMs

While (bio)degradation and persistence is mainly explored in the case of organic and carbon-based NMs, not many studies exist with respect to metallic NMs. This is due to the fact that metals cannot be degraded, although the dissolution and speciation of metals may be affected by abiotic and biological interactions yielding changes in their environmental availability [65]. As Stone et al. [65] suggested, metallic NMs may dissolve and release ions into solutions leading to persistence, which is similar to that of traditional contaminants.

For the study of metallic (Ag, TiO_2, CeO_2) NMs O'Brien and Cummins [66] proposed a three step semi-quantitative risk assessment framework for assessing the environmental concerns of metallic NMs during aquatic exposure and behaviour, based on basic aggregate distribution data from literature. The framework considers the released surface water quantities of NMs and predicts their environmental behaviour using available information on the volumes produced per year, estimation on their release in surface waters and other environmental compartments. These calculations are directly linked to the persistence of NMs by their likely state (and fate) in the environments presented in the study and ranked in different persistence categories [66]. The characteristics used for estimating the persistence of metallic NMs were primary size, aggregate size, point of zero charge, the surface coating, its solubility, the density, as well as environmental factors like the ambient pH, DOC (humic acid), Ca^{2+} concentration, the fresh-water depth and the speed of the river tested. The resulting model ranked the persistence of NMs in 4 categories:

- Ranking 1: > 0.1%

- Ranking 2: 0.1% – 1%
- Ranking 3: 1% – 10%
- Ranking 4: 10% – 100%

The persistence of Copper-based (Cu, CuO, CuS) NMs were tested by Kent and Vikesland using a dissolution kinetics model employing glass substrates loaded with copper NMs and exposed in real environmental conditions in Stroubles Creek in Blacksburg, Virginia [67]. Similarly, the persistence of Ag NMs was studied in synthetic seawater (SW) conforming to ASTM International standard D1141, Moderately Hard Reconstituted Water (MHW) according to EPA guidelines [68] and stock solutions of Suwannee River Standard I and Standard II, using DLS, UV-Vis and AFM [69].

In general, several studies exist on the (bio)degradation of inorganic NMs for therapeutic purposes. Feng et al. [70] used Prussian blue and nuclear fast red staining of liver and spleen sections to demonstrate that the degradation and removal of PEI-coated IONPs was faster than that of PEGylated IONPs suggesting a coating degradation effect. Rengan et al. [71] demonstrated the enzymatic degradation potential of liposome-surface-modified gold NMs in Swiss albino mice.

4.1.4 Identified gaps for NMs (bio)degradation and persistence

Based on the literature search performed, so far, a few gaps and challenges regarding the (bio)degradation and persistence of NMs were identified. Here these gaps are presented and relevant literature that include:

- Enzymatic degradation is the most studied mechanism of degradation. But cell and bacterial degradation need to be studied further. And all need to consider the role/impact of the biomolecule corona / competitive interactions.
- Characterizing intracellular transformations (e.g., intracellular dissolution of Ag NPs) is also necessary to assess bioavailability - detailed analysis requires synchrotron-type experiments.
    - Research on cell and bacterial degradation of NMs already exists, although enzymatic degradation has been studied a lot more. In general neutrophils and macrophages are those that were found able to degrade NMs [5], [72]–[74], as well as human osteosarcoma cell lines (MNNG/HOS) [75]. The main mechanisms for degradation by neutrophils is by endocytosis and extracellularly by producing extracellular trapping networks containing proteases such as MPO [5], [74] and through a NET (Neutrophil Extracellular Trap) [73]. Similarly, the intracellular transformation and speciation of NMs has been studied using X-ray absorption spectroscopy (XAS) [76]–[83] and microscopy techniques [84]. Another approach by Sun et al. used Based AIEgen ((9-anthrylmethyl)bis(2-pyridylmethyl)amine) (APA) and ICP-MS analysis to monitor the intracellular degradation and dissolution of ZnO NMs [85], [86].
- The exact mechanism of enzyme-catalysed biodegradation remains ambiguous.
    - Going back to enzymatic degradation, not many studies exist regarding the exact mechanism of action of the process. Srivastava et al. [87] studied the biodegradation of carbon dots and reported that they undergo peroxide catalysed degradation in the presence of lipase. The degradation process is based on the introduction of defects on the surface of the NMs [88]. Differently charged carbon dot species exhibit unique degradation kinetics upon being subjected to enzyme oxidation and this decomposition correlates with the relative accessibility of the

enzymatic molecule [87]. The peroxidase activity has also been emphasised in a review by Vlasova et al. [21] via the activation of the "dormant" peroxidase activity of hemoproteins by the nano-surface. The peroxynitrite-driven pathways realized in macrophages via the engagement of NADPH oxidase- and NO synthase-triggered oxidative mechanisms is also reported [21]. The degradation process can be enhanced by the presence of specific functional molecules that can enhance the catalytic activity of horseradish peroxidase (HRP) and xanthine oxidase (XO), like azido coumarins and cathecol derivatives, which are good reducing substrates and strong redox mediators [19].

- The extent and mechanisms of NPs uptake by plants in real soils and subsequent translocation remain to be clarified.
    - With respect to environmental studies, Wang et al. [89] stated that limited research exists on the fate and degradation of carbon-based NMs, which leads to lag in industry growth. During their study, Wang et al. demonstrated the ability of Labrys sp. bacteria to degrade carbon-based NMs, which they could use as a carbon source to support growth. This was achieved via aerobic biodegradation of carbon-based NMs through an extracellular biogenic Fenton-like reaction [89]. This is also linked with the gap on limited research regarding the extent and mechanisms of NMs uptake by plants in real soils and subsequent translocation and persistence. The fate of NMs inside plants is strongly correlated with their characteristics and plant species [90]. Nanomaterials can follow the apoplastic and/or the symplastic pathways for moving up and down the plant, and radial movement for changing from one pathway to the other [90]. Several mechanisms have been proposed for the internalization of nanoparticles inside the cells, such as endocytosis, pore formation, mediated by carrier proteins, and through plasmodesmata." Furthermore, when released into the soil NMs can interact with microorganisms and compounds, which might facilitate or hamper their absorption [90], [91]. Previous studies have shown higher uptake and persistence of NMs compared to bulk chemicals in plants [92], while Montes et al. [93] reported that there are currently no standard protocols for the quantification and characterisation of NMs in plant tissues, which leads in conflicting data and inhibits extensive study on their degradation and persistence. As suggested, combining imaging and chemical techniques can help decode the plant uptake mechanism and study the properties of NMs that can change as they travel through it. Synchrotron based X-ray fluorescence mapping and X-ray absorption spectroscopy(SR-XFM/XAS) are techniques that quickly gain popularity due to their ability to provide elemental composition, localisation and chemical speciation of NMs in plants [93]. Most studies reporting NMs degradation within plants have dealt with inorganic NMs and have monitored their dissolution and speciation with respect to NMs transformation [94]–[98]. Experiments with crop plants have demonstrated that some NMs, e.g., TiO_2, are taken up by roots and translocated to aboveground tissues, including fruits, without biotransformation [99], [100], while CeO_2 can remain as NM, but also releases Ce ions that can be incorporated into organic compounds [99], [101]. On the other hand, ZnO ENM is transformed at the soil/root interface, leading to tissue Zn enrichment. Overall, most ENMs are taken up by plants with either low or no transformation, and accumulate in tissues" [99].

- Pressing need to develop monitoring devices capable of measuring those aspects of engineered nanomaterials that result in biological responses in humans.
    - One report on the CB Nanotool, which estimates the presence and persistence of NMs in working environments based on their specific characteristics. The tool was based on ISO/TS 12901-2:2014 [102].

- Electrochemical, optical and mass sensitive sensors have been proposed for the detection of quantum dots, silver and gold NMs [103].
- The concentration of titanium in EBC may serve as a direct exposure marker in workers producing TiO_2 pigment [104].
- Devices like Fast mobility particle sizer (FMPS), Electrical low-pressure impactor (ELPI), aerosol particle mass analyser (APMA), and Tapered element oscillating microbalance (TEOM) [105].
- Markers of oxidative stress could be used for the creation of monitoring devices [104].
- Five different monitors for measuring personal exposure to airborne nanomaterials are commercially available: (1) the Miniature Diffusion Size Classifier DiSCmini (Testo, Titisee-Neustadt, Germany, identical with miniDiSC), (2) the Aerasense NanoTracer (oxility, Eindhoven, the Netherlands), (3) the partector (naneos, Windisch, Switzerland), (4) the Personal Ultrafine Particle Counter (PUFP C100 and C200, Enmont, New Richmond, OH; USA) and (5) the MicroAeth AE51 (AethLabs, San Francisco, CA, USA)." [106]
- Nanotracers and nanomonitors can have diverse applications such as air quality monitoring, environmental monitoring and nanoparticle exposure assessment [107].
- Using RNA sequencing and computational approaches [108], [109].
- Using an *in vitro* air–liquid interface (ALI) exposure system [110].
- *In situ* monitoring of ROS-mediated MWCNT degradation by liquid-cell transmission electron microscopy [30].
- Biomonitoring of *in vivo* and *in vitro* NM-induced genotoxicity studies, using an assay that scores cytotoxic events from apoptotic or necrotic cell ratios [111].
- The need to assess the human and environmental risks of nanoscale materials has prompted the development of new metrological tools for their detection, quantification and characterisation. Some of these methods have tremendous potential for use in various scenarios of nanotoxicology. However, in some cases, the limited dialogue between environmental scientists and human toxicologists has hampered the full exploitation of these resources [112].

- Need for relevant information on airborne NMs and nanostructured particles, in an efficient and cost-effective way.
  - There are two distinct categories of measurement techniques to characterise the aerosol; the first is based on the collection of aerosol particles on a substrate, and the second is on in situ, near real-time measurement of aerosol [113].
  - The effective density of airborne NMs can be measured using microfluidic devices [114].
  - A Micro-Electro-Mechanical Systems (MEMS)-based condensation particle growth chip for optically measuring the airborne nanoparticle concentration has been proposed [115].
  - Airborne nanoparticles can be collected with low-cost surfactants like sodium

dodecyl sulphate or chemical reagents like ammonium molybdate and ascorbic acid [116].

- Efficient collectors of NMs (solution-blown 20–50 nm nanofibers), based on van der Waals forces [117].
- Prediction of airborne NMs at roadside location using a feed–forward artificial neural network [118].
- An air–liquid interface exposure system for assessing toxicity of airborne NMs [119].
- Near real-time, field-portable instrument for selective quantification of airborne CNT concentration, using Raman spectroscopy [120].
- A methodology for high efficiency retrieving and accurate quantification of airborne magnetite NMs [121].
- A first-of-its-kind aerosol exposure device for toxicity testing, referred to as the Dosimetric Aerosol *In Vitro* Inhalation Device (DAVID) [122].
- There is a research gap in standardized methods to assess the exposure to airborne NMs in different environments. The controllable generation of nanoparticle aerosols has long been a challenging objective for researchers and industries dealing with airborne NMs [123].
- A versatile generator of NMs aerosols, to use it for the measurement of exposure to airborne nanoparticles [123].
- A microfluidic condensation nanoparticle counter using water as the condensing liquid for assessing individual exposure to airborne nanoparticles [124].
- A nano aerosol chamber for *in vitro* toxicity (NACIVT), a portable instrument for realistic safety testing of inhaled NP *in vitro* and evaluated effects of silver (Ag) and carbon (C) NMs [125].
- Analysis with molecular dynamics simulation of nanoparticles with PS (pulmonary surfactant) bio corona [126].

- Need for relevant information on water borne NMs and nanostructured particles, in an efficient and cost-effective way.
  - One related paper about accelerated degradation of water-borne acrylic nanocomposites used in outdoor protective coatings. It is rather more related to the study of NMs degradation [127].

- The identification of biomarkers associated with exposure to various types of NMs will allow monitoring of exposure of internal dose.
  - Omics approaches using the engineered nanomaterials-based alterations in gene and protein expression [128].
  - Urinary and white blood cell (WBC) 8-hydroxydeoxyguanosine (8-OHdG), and exhaled breath condensate (EBC) 8-isoprostane as oxidative stress biomarkers, as well as WBC global methylation [129].
  - Myeloperoxidase as a biomarker for the ranking of pulmonary toxicity of NMs [130].

- Lung damage markers (SP-D and pulmonary function), cardiovascular disease markers (VCAM-1, ICAM-1, LDL, and TC), oxidative stress markers (SOD and MDA), and inflammation markers (IL-8, IL-6, IL-1β, TNF-α, and IL-10) were associated with occupational exposure to nano-TiO_2 [131].
- Biomarkers associated with at least three CNT/F (carbon nanotubes/nanofibers) metrics were 72 kDa type IV collagenase/matrix metalloproteinase-2 (MMP-2), interleukin-18, glutathione peroxidase (GPx), myeloperoxidase, and superoxide dismutase (SOD) in sputum and MMP-2, matrix metalloproteinase-9, metalloproteinase inhibitor 1/tissue inhibitor of metalloproteinases 1, 8-hydroxy-2'-deoxyguanosine, GPx, SOD, endothelin-1, fibrinogen, intercellular adhesion molecule 1, vascular cell adhesion protein 1, and von Willebrand factor in blood [132].
- Immune System Biomarkers Produced by RAW 264.7 and Human Whole Blood Cell Cultures, caused by graphene oxide nanoparticles [133].
- CeO_2, SiO_2 and CuO metal oxide NMs on HepG2 cells caused decreased nucleotide concentrations coupled with increased concentrations of nucleic acid degradation products [134].
- Five miRNAs and two proteins were proposed as specific exosomal biomarkers for the exposure of HEK293 cells to PbS–MPA quantum dots [135].
- Markers like MDA, HNE, HHE, C6–C10, 8-isoprostane, 8-OHdG, 8-OHG, 5-OHMeU, 3-ClTyr, 3-NOTyr, o-Tyr and C11 were elevated in exhaled breath condensate of workers exposed to NMs during iron oxide pigment production [136].
- Interleukin 8 secretion and Prostaglandin E2 decrease are both biomarkers for Pd-NPs (palladium NMs) [137].
- Condensate (EBC) titanium and markers of oxidation of nucleic acids (including 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 5-hydroxymethyl uracil (5-OHMeU)) and proteins (such as o-tyrosine (o-Tyr), 3-chlorotyrosine (3-ClTyr) and 3-nitrotyrosine (3-NOTyr)) were analysed from samples of their exhaled breath [104].
- Response to CdS QDs (quantum dots) of genes related to apoptosis (AIFM2 and APAF1), oxidative stress response (OXR1 and AOX1) and autophagy (ATG3 and ATG7), as potential biomarkers. Other possible biomarkers specific for mitochondria function are LONP1 andHSPD1 [138].
- Utility of a CSC-based (Cancer Stem Cells) assay as a predictive screening tool for carcinogenicity testing of nanomaterials, and identifies potential biomarkers that can be used for safe-by-design strategies of NMs [139].
- The BRCA2, CYP1A1, CYP1B1, CDK1, SFN and VEGFA genes were observed to be upregulated specifically from increased CdSe exposure and suggests their possible utility as biomarkers for toxicity [140].
- Increased KL-6/TGF-β (fibrosis biomarkers) levels in the biofluids of MWCNT (multi-wall carbon nanotubes)-exposed workers. Inflammatory cytokines content was also increased in these workers [141].
- Significant upward trends for immune markers C-C motif ligand 20, basic fibroblast growth factor, and soluble IL-1 receptor II, with increasing exposure to multi-walled carbon nanotubes (MWCNT) [142].
- Co-regulated miR-mRNA cluster could represent potential biomarkers of sub-toxic metal-based nanoparticle exposure [143].
- The study on interactions of ENMs with the gut microbiota might be useful for the identification of new biomarkers [144].
- Forty-eight differentially expressed genes (DEGs) (related to asthma) may represent biomarkers to a potentially large variety of metal/metal oxide NMs [145].

  - Antioxidant enzymes catalase, glutathione S transferase, glutathione peroxidase and glutathione reductase activities were significantly elevated along with significant decrease in superoxide dismutase activity in treated rat organs [146].
  - Non-invasive biomonitoring using markers of oxidative stress, LTB4 and LTE4 may be most useful and could be recommended biomarkers for preventive examinations and monitoring of workers with occupational exposure to NMs [147].

- Need for design of new and suitable tests for NMs. For example, enzymatic degradation studies typically lack consideration of the competitive role of the biomolecule corona which influences accessibility of enzymes to particle surface.

  - Many existing OECD test guidelines are suitable for nanomaterials and consequently, hazard data collected using such guidelines will fall under OECD's system of Mutual Acceptance of Data (MAD) which is a legally binding instrument to facilitate the international acceptance of information for the regulatory safety assessment of chemicals [148].

  - NanoCRED: A transparent framework to assess the regulatory adequacy of ecotoxicity data for nanomaterials [149].

  - Genotoxicity is a hazard endpoint required in all product regulations (REACH, biocides, pharmaceuticals, medical devices, food additives, cosmetics, etc.), as well as one of the categories considered within the United Nations' Globally Harmonized System (GHS) for Hazard Communication [150].

  - Mesocosm testing: a method consisting in monitoring the evolution of a re-created miniature ecosystem following nanomaterial contamination [151].

  - A new tool for the long term *in vitro* ecotoxicity testing of nanomaterials using a rainbow-trout cell line (RTL-W1) [152].

  - The CT (Cell Transformation) assay may be useful for screening the carcinogenic potential of MWCNTs and TiO_2 NMs [153].

  - Novel tools can be based on combination of multimodal spectroscopies to study cellular uptake processes [154].

  - A hybrid quantitative multi-nano-read-across approach that combines interspecies correlation analysis and self-organizing map analysis [155].

  - High throughput toxicity screening and intracellular detection of NMs [156].

- Research should focus on both aspects of a QSAR study: the generation of nano-specific theoretical descriptors and experimental test data.

  - While the use of predictive and structural modelling has been suggested [157], [158] for the risk assessment of NMs, including (bio)degradation and persistence relevant studies were identified.

- Even though many different studies have been performed regarding the biological behaviour of inorganic nanoparticles, long-term *in vivo* data is still scarce, limiting the capacity to evaluate the proposed NMs for clinical use.

  - Partial surface PEGylation could prolong the blood circulation and increase the tumor accumulation of ultrasmall nanoparticles to a maximum extent, in tumor-bearing mice [159], [160].

- Demonstration of biodegradable inorganic mesoporous nanosystems with specific biodegradation behaviour sensitive to tumour microenvironment [161].
- Ultrapure laser-synthesized Si-based nanomaterials, as safe and biodegradable nanomaterials for clinical use [162].
- The aqueous dispersion of Fe_3O_4/salicylic acid nanoparticles intravenously injected in chick embryos was found as biocompatible and with no embolic risk even after repeated magnetically aggregation. It showed a dose-dependent bloodstream time persistence and were stored mainly in the liver [163].
- Tailor made Iron oxide nanoparticles (IONPs) coated with polyethylene glycol (PEG), were found to be completely retained at a tumor site. (they were used for magnetic resonance molecular imaging) [164].
- Folate-graphene chelate manganese nanoparticles as a theranostic system for colon cancer MR imaging and drug delivery [165].
- Size reduction to ultrasmall nanoparticles (USNPs) is a suitable approach to promoting metal excretion by the renal pathway [166].
- Another paper reported mesoporous silica nanoparticles as a multifunctional controlled drug delivery nanoplatform for infectious diseases treatment [167].
- Another study was about the therapeutic outcomes of ceria nanospheres (Ceria NSs) and ceria nanorods (Ceria NRs) in an *in vivo* study of mild traumatic brain injury [168].

- Need to recover the NMs from environmental and biological matrices and measure the properties of these partially transformed NMs, or to measure them *in vivo*/*in situ*.
  - While much work is being performed in the field, there are still no standardised methodologies for the retrieval of NMs from complex environmental matrices [169]–[172]. Attempts have focussed on the quantification of NMs in complex matrices [173], [174], the separation, detection and characterization of NMs in municipal wastewaters using hydrodynamic chromatography coupled to ICP-MS and single particle ICP-MS [174], functional assay-based strategies for nanomaterial risk forecasting [12], the use of nanoparticle tracking analysis (NTA) as the online detector for asymmetric flow field-flow fractionation (FFF) [175] or FFF and UV spectroscopy coupled with ICP-MS and LCMS/MS [176] and a number of other techniques that have been already been used for the monitoring of the NMs' degradation and persistence [177]–[179].
  - Analytical tools that potentially can facilitate elucidation of key NM characteristics, such as ion beam microscopy (IBM) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) have the potential to advance the understanding of biopersistent NM kinetics [180].

- Detection and quantification of NMs, especially determination of their state, i.e., dissolution, aggregation, and agglomeration within biological matrices and other environments are still challenging tasks; moreover, mechanisms of nanoparticle (NP) translocation and persistence remain critical gaps.
  - Two of the reviewed studies reported results on the persistence of the studied NMs; One of these two studies, regarding MoS_2 nanosheets, showed that this type on NMs does not show persistence in living systems and natural waters [181]. While the other study reported that the environmental persistence of nanosilver

is likely, due to its elevated use and release [182].

- A study reported that no structural changes or degradation of few layer graphene (FLG) and graphene oxide (GO) nanomaterials were detected in a simulated oral ingestion, suggesting that they are biopersistent when administered by oral route [183].

- Three of the reviewed papers referred explicitly on the biodegradation and biopersistence of NMs, in addition to their translocation [184]–[186]. Two of them investigated the biodegradation of graphene oxide nanosheets in the brain [184] and TiO_2 NMs in different organs in rats [186], that have been translocated through inhalation.

- A variety of techniques for the study of the translocation of NMs, like Raman spectroscopy and confocal microscopy, as well as advanced imaging techniques. The reviewed papers clearly show that the development of different or new and innovative techniques for the study of translocation and the route and the fate of NMs inside an organism, is continuous [187]–[197].

- Gaps exist in test methods for environmental fate, such as methods to estimate heteroagglomeration and the tendency for MNs to transform in the environment.

  - There is an OECD draft guideline to measure agglomeration of nanoparticles (OECD, 2016b) [198], [199]. The proposed functional assay in this guideline measures homoaggregation of nanoparticles in an aqueous suspension [198].

  - Relevant studies exist regarding the heteroagglomeration of TiO_2 NMs and bulk and three different microalgae species, and under different environmental conditions (freshwater and marine water) [200], [201]. The heteroagglomeration process was examined by means of co-settling experiments and the Derjaguin-Landau-Verwey-Overbeek (DLVO) approach.

  - A second method to monitor agglomeration behaviour (homoagglomeration, heteroagglomeration and deposition) of NMs is to calculate surface affinity from an aggregation experiment.

  - Heteroagglomeration can be a predictive parameter for toxicity to environmental species such as algae. The BAuA research project (nanoGRAVUR) tested an approach based on curvature analysis and oscillatory measurement [202].

  - Darkfield microscopy and hyperspectral analysis as two methods proposed to investigate the fate (including heteroagglomeration) of silver nanoparticles in wastewaters [203].

  - A predator-and-prey-based laboratory microcosm was established using *Paramecium caudatum* and *Escherichia coli* to evaluate the effects of $nTiO_2$. The surface interaction of $nTiO_2$ with E. coli significantly increased after the addition of Paramecium into the microcosm. This interaction favoured the hetero-agglomeration and co-sedimentation of $nTiO_2$ [201].

  - Water chemistry had profound effects on aggregation, dissolution, and algal toxicity of nanoparticles. The strongest homoaggregation was associated with the highest ionic strength, but no obvious correlation was observed between the homoaggregation of nanoparticles and pH or dissolved organic matter content of the water samples [198].

  - Nanoparticle tracking analysis (NTA) can measure individual particles to create a

size distribution and measure the particle number. The suitability of the method was also investigated for analysis of engineered NMs in complex matrices by measuring their agglomeration and sedimentation in municipal solid waste incineration landfill leachates over time [204].

- Another paper reported Quantitative Property-Property Relationships for the study of the NMs transformation, including heteroagglomeration [205].
- Another study investigated the stability of co-existing ZnO and TiO_2 NMs in natural water, and in particular, their aggregation, sedimentation, and heteroagglomeration mechanisms [187].

- The practical requirements for bioaccumulation testing of NMs have been given less attention.
  - Many of the reviewed papers investigate the uptake and the (bio)accumulation of NMs in a variety of organisms, from microalgae and algae, to crustaceans, invertebrates, fish, plants, snails, and mammals. Many of the mentioned organisms are in aquatic environments. Also, a good number of papers refer to the so called "microcosms", that was also reported in the literature review for another research gap [206]–[211].
  - Several papers compare (bio)accumulation between a NM and its equivalent bulk material [212]–[216].
  - Some papers report important or even toxic (bio)accumulation, while others not a toxic one [210], [211], [217]–[219].
  - Several papers claim that there is uptake of NMs at the first level of a food chain, but they do not accumulate in subsequent levels of the chain [220]–[222].

- Close the knowledge gaps around the latent animal exposure and the life cycle analysis of 2D material-based products properly.
  - Extensive research exists regarding green chemistry metrics and the LCA of NMs. On the other hand, databases and tools to facilitate cross comparisons of these sustainability indicators for timely decision making during the route selection phase of development do not currently exist [223]. A major challenge to implementing LCA for NMs and nanoproducts continues to be a lack of data or large data repositories. Even with data, there is a need to construct meaningful insights as well as include efforts to validate reports of existing data and understand the influence of variation in LCA or methods that improve upon LCA. Although several LCAs on NMs or nano-enabled products have been published [224], few systems have been developed to analyse LCA for nanotechnology. LearNano [225] is one system that estimates the release of engineered NMs across the entire life cycle. However, this represents only compartmentalization of NMs, a single lens of nanomaterial knowledge. To better account for hazards in addition to environmental impact, hybrid life cycle assessment and risk assessment (RA) approaches have been proposed [226]. There are four schools of thought in relation to combining LCA and RA. The emergent hybrid RA-LCA schools of thought are: RA for LC-hotspots (a full LCA is performed then RA is applied to identified 'hotspots'), Combining Results (results of a RA and LCA are combined, often with use of a multi-criteria decision making method), Chain Perspective (makes use of a chemical life cycle versus the product basis commonly used in LCA, also combines all chemical impacts in a geographic area), and Knowledge Integration (makes use of parts of RA during impact assessment stage of LCA). Each school of thought has strengths and weakness with trade-offs in aspects of RA or LCA

[226].

- 2D-materials undergo peroxide catalysed degradation in the presence of lipase. Differently charged 2D species exhibit unique degradation kinetics upon being subjected to enzyme oxidation. Furthermore, this decomposition correlates with the relative accessibility of the enzymatic molecule [227].
- The GaBi software has been used for the LCA of graphene [228].

4.1.5 Techniques used for the study of NMs (bio)degradation and persistence

With respect to the techniques used to characterise the NMs and monitor their degradation, a number of established techniques have been used as demonstrated in Table 3 [18].

Table 3. Experimental and molecular simulation methods used for the monitoring of NMs degradation. Table reprinted and adapted from [18].

Method	Reference
Scanning Electron Microscopy (SEM)	[23]–[25], [31], [42], [50], [52], [55]
Transmission Electron Microscopy (TEM/STEM)	[19], [20], [23]–[25], [30]–[33], [38], [39], [43], [44], [47], [49], [50], [59], [67], [71], [79], [81], [82], [84], [86], [87]
Raman spectroscopy	[19], [20], [23], [25], [31], [33], [39], [41]–[44], [47], [49]–[52]
Vis–NIR spectroscopy/UV–Vis–NIR spectra	[20], [25], [31]–[33], [41]–[44], [47], [50]–[52], [69], [87]
Electron spin resonance (ESR) spectroscopy	[25]
Atomic Force Microscopy (AFM)	[33], [42], [43], [49], [51], [52], [67], [69], [87]
^{14}C labelling	[24]
Liquid chromatography–tandem mass spectrometry (LC-MS/MS)	[24], [25], [33]
Gas chromatography–mass spectrometry (GC-MS)	[24], [25], [28]

Electron paramagnetic resonance (EPR) spectroscopy	[33], [44]
Electrospray ionization–mass spectrometry (ESI-MS)	[44]
Circular dichroism spectroscopy	[42]
Attenuated total reflectance–IR (ATR-IR) spectra	[23]
X-ray diffraction (XRD)	[23], [33], [50]
Fourier-transform infrared spectroscopy (FTIR)	[31], [33], [39], [50]–[52], [55], [57]
X-ray photoelectron spectroscopy (XPS)	[31], [50]–[52], [67], [87]
Energy-dispersive X-ray spectroscopy (EDS)	[50], [67], [79], [81]
Oxygen flask combustion (OFC)	[50]
Thermo-gravimetric analysis (TGA)	[39], [50]
Column transport experiments (CTE)	[51]
ICP-OES/ICP-AES/ICP-MS	[67], [71], [79], [84], [85], [87], [229]
Dynamic Light Scattering (DLS)	[33], [39], [51], [52], [55]–[57], [59], [69], [87]
Chlorination and irradiation	[52]
Total organic carbon (TOC)	[52], [67]
Inorganic carbon (IC)	[52]
In vivo degradation (rat exposure)	[30], [31], [33], [71]
In vitro degradation (cell-line exposure)	[30]
Fluorescent microscopy	[31], [33], [87]

Native polyacrylamide gel electrophoresis (PAGE)	[39]
Densitometry analysis	[39]
Micro- and mesocosms	[28]
Dissolution	[67], [85], [229]
Quartz microbalance	[57]
Phosphomolybdic acid (PMA) assay according to the Stevenson method	[59]
Nanoparticle tracking analysis (NTA)	[56]
Synchrotron analysis	[76]–[83]
Magnetometry	[81]
Molecular docking	[39], [41], [42], [47], [230], [231]
Molecular dynamics simulation	[35], [39], [230], [232]
Homology modelling	[47]
Derjaguin–Landau–Verwey–Over-beek (DLVO) theory	[51]

4.1.6 Comparison of NMs (bio)degradation and persistence with other chemicals

In general, there is similarity of the methods used to test the (bio)degradation and persistence of NMs with other chemicals, e.g., bulk and organic chemicals. Same as with NMs, enzymatic, bacterial and fungi degradation can be very useful or (bio)degradation of chemical substances and compounds [233]–[236].

Other methods of degradation of chemicals that were reported [234], [237]–[243], were:

- Photocatalytic degradation (using either UV or visible light)
- Sonochemical degradation
- Oxidation methods and advanced oxidation methods, such as: ozonation, UV/H2O2, UV/PDS, Fenton process/reaction
- Analysis of formation of NER (non-extractable residues)
- Isotope tracer compounds for tracing compound turnover in complex environments

With respect to the analysis methods reported [238]–[241], [243]–[246], these include:

- NMR analysis
- FTIR analysis
- Time-of-flight high-resolution mass spectrometry (TOF-HRMS)
- XRD
- TEM (Transmission Electron Microscopy) observation
- GC-MS
- Electron energy-loss spectroscopy (EELS)
- TOC

Most of these methods have also been used in the case of NMs, as presented in section 3. Same as in the case of NMs, the use of computational techniques is also proposed when experimental studies are difficult or impossible to be performed [236], [247], [248].

Lowry et al. [249] attempted to define the NMs-related parameters that correspond to those used for studying the fate of organic chemicals in different environmental compartments and the intermedia distribution. These are presented in Table 4, and it is evident that some deviation exists when considering the different types of substances.

Table 4. Properties of nonpolar organic chemicals and the environmental compartments used to predict their partitioning in the environment and potential corresponding parameters for engineered NMs. Table reprinted by [249].

Intermedia distribution	Parameter(s) used to predict distribution in the environment	
	Nonpolar organic chemicals	Engineered nanomaterials
Pure compound–water	aqueous solubility, C_w^{sat}	Agglomeration state, dispersion stability (ratio of sedimentation flux to diffusion flux)
Air–water	Henry's law constant, K_H	N/A†
Water–solid	K_d (function of K_{ow} and mass fraction of organic carbon in soil/sediment [f_{oc}])‡	Attachment efficiency (α), ionic strength, ionic composition, f_{oc}, pH, shear, exchange site density and surface charge of solid surface

† N/A, no analogous concept for particulate ENMs.

‡ K_{ow}, *n*-octanol-water partition coefficient.

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While a single OECD guideline for the stability of NMs exists (OECD TG 318 Dispersion Stability of Nanomaterials in Simulated Environmental Media) [199], [250], Steinhauser and Sayre [251], from the ProSafe project, have provided a good overview on the methods that can be used to test the persistence of NMs. With respect to biodurability, *in vivo* and *in vitro* methods that are applicable to mammalian fluid, tissues and cell cultures exist, but are not currently validated, while dissolution in physiological fluids, environmental dissolution are currently under development withing the OECD. It is also suggested that environmental biodegradation for carbonaceous materials may be assessed via adaptation of existing OECD biodegradation test guidelines. For zeta potential there is a lack of clear reporting guidelines, while the measurements can be easily affected by the solution's pH, ionic strength, the NMs coating (e.g., organic coatings) and interferences due to media-induced agglomeration or electrode blackening. To make these measurements scientifically useful it is suggested to report relevant electrophoretic methods complemented by their respective metadata, including the identification and reporting of the NM's isoelectric point, as it can be more comparable across materials [251].

The density of NMs can be studied using gas pycnometry (for powders) and analytical centrifugation in water. While both methods are reliable, the former requires a large sample size for analysis and the latter is expensive and not available in most cases. The authors state that a new benchtop centrifugation method appears reliable but is not validated and the current results are bases on a small dataset. In any case, the benchtop method is less cost- and time-intensive and more readily accessible [251].

Regarding persistence and workplace exposure, the rotating drum and the continuous drop

methods are standardised and available, although they are sensitive to the presence of moisture. In this case, the revision of EN 15051 [252] is proposed [251].

For the dispersion stability of NMs the OECD adopted test guideline [199] appears to be reliable and relevant to NMs, although it provides a simple classification and does not address heteroagglomeration, for which no methods have been developed. Time-resolved DLS methods can provide direct measures of attachment efficiency and can be adapted to the existing OECD protocol. Finally, in the case of surface affinity, no standardised protocols or test guidelines exist for aggregation and heteroaggregation [251].

In general, a wide range of standardised methods exist for the study of persistence and degradation of bulk chemicals. These include:

- A weight-of-evidence (WoE) approach
- A multi-media (air, water, soil) fate model to estimate the distribution and degradation of chemicals released to the environment
- OECD 301: Ready biodegradability tests (RBTs)
- OECD 302: Inherent biodegradability
- OECD 303: Aerobic sewage treatment
- OECD 304: Inherent biodegradability in soil
- OECD 306: Biodegradability in seawater
- OECD 307: Aerobic and Anaerobic Transformation in Soil
- OECD 308: Aerobic and anaerobic transformation in aquatic sediment systems
- OECD 309: Aerobic and anaerobic transformation in surface water
- OECD 310: Ready Biodegradability - CO_2 in sealed vessels (Headspace Test)
- OECD 311: Anaerobic Biodegradability of Organic Compounds in Digested Sludge: by Measurement of Gas Production
- OECD 314: Simulation biodegradability tests
- US EPA OPPTS 835.3140: Aerobic biodegradation
- Life Cycle Analysis (LCA)
- Life cycle inventories (LCIs)
- Cradle-to-grave approach

Several of these approaches have been tested for their applicability in the study of the degradation and persistence of NMs, but substantial data are lacking for tests in terms of tests performed according to OECD guidelines. Tests for ready biodegradability (OECD TG 301 tests) may face practical obstacles for certain MNs (e.g., due to the need for relatively high concentrations of dissolved organic carbon (approximately 20 mg DOC/L)), test for inherent biodegradability (OECD TG 302 tests) and simulation tests (OECD TG 314) are also lacking [253]. Furthermore, Handy et al. mention that while numerous regulatory ecotoxicity tests use bacteria in the test matrix as part of microbial biodegradation assessment (OECD 301, 302, 304), these

would seem appropriate for carbon-based NMs, which presumably could be metabolised eventually to carbon dioxide and water. However, the tests would be inappropriate for metal ENMs that are already in an elemental state [254], although a recent study used TGs 301 and 310 to evaluate the effect of biogenic AgNPs on total heterotrophs aerobic microorganisms [255].

A single study on the applicability of the OECD TG 303 in TiO_2 NMs concluded that the guideline is applicable for the testing of nanomaterials if modifications regarding the dosage, nitrifying conditions, and a characterisation of the nanoparticles in the effluent are applied. A compilation of the cumulative mass balance by comparison of the total dosage added with the amount in the outflow and in the activated sludge is recommended [256]. Another study has looked into the aerobic and anaerobic biodegradation of bio-based cellulose nanomaterials for commercialisation, using the OECE TG 311 and the US EPA OPPTS 835.3140, without mention of any modifications [257].

The OECD TG 307 has been used for the study of graphene mineralisation in soil [258], without any modifications, which suggests its applicability for NMs, but Baun et al. mention that as this TG is intended for soluble chemicals, it may have limited applicability in NMs [253].

Similarly, the rest of the methods have been used for the testing of NMs (bio)degradation and persistence:

- A weight-of-evidence (WoE) approach [259], [260].
- A multi-media (air, water, soil) fate model to estimate the distribution and degradation [261], [262].
- Life Cycle Analysis (LCA) [263], [264].
- Life cycle inventories (LCIs) [265], [266].
- Cradle-to-grave approach [267], [268].

4.2 Extensive literature search on safe by design for nanomaterials

The ELS provided an extended number of studies with respect to the safe by design (SbD) of NMs. Out of all the papers screened, the most significant and of highest quality are reported here. Furthermore, the study for each presented section was interrupted, following the screening of a substantial number of papers that were considered as repeating the information already extracted.

Research included papers, reviews, and reports in the public domain for which Novamechanics had full text access. To retrieve the relevant literature, the following list of search terms combinations was formed, based on the questions that this report is trying to answer:

1. ("safe by design" OR SbD) AND definition AND nanomaterials
2. ("safe by design" OR SbD) AND strategies AND nanomaterials
3. ("safe by design" OR SbD) AND boundaries AND existing AND strategies AND nanomaterials
4. ("safe by design" OR SbD) AND strategies AND nanomaterials AND (substances OR chemicals)
5. ("safe by design" OR SbD) AND existing AND (methods OR strategies) AND nanomaterials AND modifications

6. ("safe by design" OR SbD) AND strategies AND nanomaterials AND (actions OR projects) OR EU

7. ("safe by design" OR SbD) AND nanomaterials AND approaches OR emerging OR computational

8. (safe OR safer) AND nanomaterials AND methodologies AND manufacturers OR (re-evaluation OR "reduced exposure" OR "reduced persistence")

9. "acceptance of nanomaterials " AND society OR "safe by design" OR SbD

10. ("safe by design" OR SbD) AND (methods OR strategies) AND nanomaterials AND products AND communication AND (societal OR society) AND acceptance

SbD definition for NMs

The ELS regarding the presence of a definition for SbD for NMs demonstrated that there has been and there is a lot of work in the field. Nevertheless, to achieve consensus on a clear and unified definition or principles seem to be challenging. There are two main reasons for the lack of consensus regarding the SbD of NMs. Firstly, the vagueness and broadness of the term "safety" [269], which is considered to be a broad and difficult to define concept, since "it is a relational value and absolute safety cannot be achieved" [269]. Safety can be studied through the notion of risk, and its constituents "hazard" and "exposure", according to the equation Risk = Hazard X Exposure. [http://optinanopro.eu/optinanopro01/files/2018/10/Safe-by-DesignProtocol.pdf]. Secondly, NMs have unique properties that differentiate them from bulk chemicals, which, so far, has make it impossible to group. As a result, it has been suggested that a case-by-case safety or risk assessment may be required for the different NMs [1-3]. This is due to potential differences in risk of the same NM considering that it ends up in different environments. This can lead to different (if any) transformations and reactivity with its surrounding environment and potential adverse effects.

Some of the early attempts for defining and studying the concept of SbD for NMs were the EU-funded NANoREG, NanoReg2 and ProSafe projects. The work performed led to the suggestion that it was more realistic to define the concept of "safer" rather than "safe" by design, which can be established using 3 pillars, namely safe products, safe use, and safe production [270] :

i) safe products by design are based on the chemical and physical properties of the NMs, such as shape, aspect ratio, crystallographic faces, and chemical additives in nano-enabled products which can maximise benefits and minimise risks
ii) safe use of NMs-containing products, including minimising waste production and safe handling and recycling
iii) safe industrial production to ensure a safe working environment as this is the most likely environment for human exposure to take place.

Later and ongoing projects and frameworks, e.g., Nanodefine [271], caLIBRAte [272], Asina, SbD4Nano, Sabydoma and SAbyNA, and SERENADE [273], have worked and are working towards the establishment of the "Safe(r) by Design" concept. From these projects, Serenade has reported that the SbD project, which is well defined in other industries, like the pharmaceutical industry, has indeed transferred to the nanotechnology domain, thanks to EU projects [273]. While this may be the case for nanomedicine, where concepts or definitions like "immunity by design" [274], and "benign by design" [275] [269] are well defined and established, it is an overstatement for the field of nanotechnology in general. The nanospecific term for the former is "nanoimmunity by design", while the latter means that the end of life of a product and aspects of it, like recovery of its components and recycling are also considered in the design phase [275]. It is an overstatement, though, to claim the same in the case of NMs in general, and therefore there is still ongoing work in the field. This is also expressed in scientific

literature, where it has been reported that SbD is not a clearly defined concept in the case of NMs [276]. The authors state that it is more like a starting point, and that in any case commercialised products will need to go through safety evaluations and regulations.

In general, the concept of safe-by-design is being considered unrealistic, as it is impossible to ensure that a product will be safe under all possible conditions. As a result, it would be more realistic to consider the concept of safer-by-design [277], which aims to minimise as much as possible the risks associated with the production and use of NMs. The concept of safer-by-design has also been central to several EU project, e.g., NanoReg2 and SUN [270]. It must be noted that currently the terms of safe-by-design and safer-by-design are currently used, in many cases, interchangeably with the exact same meaning.

SbD for NMs can be based on established principles like [https://epub.oeaw.ac.at/0xc1aa5576_0x003aa569.pdf]:

- Design for safety: identify particularly high-risk areas and to minimise both probabilities of occurrence and effects, with systems being analysed through all phases of their life cycle.
- Green chemistry: development of products with lower environmental risk, including the inclusion of ecotoxicity tests in it [278].
- Ecodesign: reduce the environmental effects of products over their entire life cycle, an aim to be achieved by means of an appropriate design during product development.
- Quality by design: introduction of quality tests during the product development process leads to a more efficient fault identification and reduction.

Based on these, the NanoTrust project proposed the development of a SbD concept for NMs based on a network of public authorities, industries, and science to achieve efficient risk management, building upon existing risk management systems and using the international standardisation for risk management (ISO 31000:2009), and ISO project management guidelines (ISO 21500:2012) [https://epub.oeaw.ac.at/0xc1aa5576_0x003aa569.pdf].

Two other concepts incorporating the concept of SbD are SIA (Safe Innovation Approach) [279] [8] [280] and AOP (Adverse Outcome Pathways) [281]. SIA is a framework incorporating both SbD and RP (Regulatory Preparedness) concepts [8] [280], while AOP encompasses both SbD (Safe by Design) and the previously mentioned "Safer by Design" concepts [281]. Besides ongoing EU-funded projects, the latest known attempt for the establishment of a safe(r)-by-design definition was through the EU NanoSafety Cluster and respective Task Force (TF), under the leadership of the former Nanotechnologies Industries Association (NIA) CEO Dr. Claire Skentelbery. In this case, the TF team had run a consultation with different field experts, but the results were not eventually published (personal communication).

4.2.1 SbD strategies available for NMs and their boundaries

As SbD has been a key topic for the study, development, and production of NMs, substantial effort has been put and is being put into field within Europe and globally. Due to their nature, properties, and methods for studying, the applied SbD strategies are usually focused to specific NMs types, e.g., metals, carbon-based etc. In the case of carbon nanotubes (CNTs) it has been suggested that decreasing their length leads to decreased toxicity, although this approach is not able to fully eliminate any adverse effects [282]. Reduction in size has been demonstrated to assist with the reduction of potential adverse effects in other NMs as well, like for example shortening TiO_2 nanoforms (e.g., tubes, fibres) [283].

In general, the surface modification of NMs has demonstrated the potential to decrease the risk originating from the use of NMs. Such approaches include the "safety by molecular design" strategy, which has been demonstrated in the case of TiO_2 NMs, following encapsulation with SiO_2 [284] or by reducing the display of silanol groups in the surface of the NM [285]. This approach resulted to decreased production of reactive oxygen species (ROS) [284] decreasing

toxicity. Similarly, the production of Au@Ag core−shell nanoparticles, without a complex surface post modification of them, making use of the Au−Ag electron compensation effect, led to more biologically safe Au@Ag core−shell NMs [286]. Similar conclusions have been demonstrated in the case of CuO NMs, where surface modification with inorganic materials led to decreased ionic release and hence bioavailability reducing toxicity [287].

Other approaches dealt with the functionalization of NMs with organic ligands to regulate their colloidal stability in different environmental media [288]. According to the authors, such methods can help with NMs categorization for the purpose of developing more detailed SbD strategies [288]. A similar approach was demonstrated in a review of *in vivo* experiments by Zhao et al. for carbon nanotubes, metal nanomaterials and quantum dots, where it was reported that non-covalent surface attachment influences NMs bioavailability and pulmonary toxicity and ultrahigh surface reactivity and resulting NMs instability lead to toxic metabolites *in vivo* [217].

Organic coating has been used in nanomedicine, for imaging purposes, of superparamagnetic iron oxide nanoparticles (SPION), which have been linked with potential risk associated with exposure, including inflammation, fibrosis, genotoxicity, and extra-pulmonary effects, all of which have been attributed to increased oxidative stress following exposure [289]. In the review by Kornbeg et al. [289] it was stated that literature is conflicting with respect to the hazardous potential of SPIONs, but in any case metanalysis showed that modifying the physicochemical properties, focussing on surface modification SBD strategies, could lead to decreased risk [289]. An equivalent study demonstrated that the formation of an artificial protein corona of bovine serum albumin (BSA), which was found to improve their biocompatibility [290].

Doping has been used to modify the properties, behaviour, and adverse effects of NMs as well. A review by Bennet et al. mentioned that substituting 10% of ZnO with Fe, led to reduced dissolution, release of Zn^{2+} and subsequently decreased toxicity. Subsequently, this approach was demonstrated to be usable in nanomedicine for reducing tumour growth in n cancer-specific toxicity in a preclinical rodent model [291]. Surface modification, as a SbD strategy has also been applied in the case of Au NMs [292] [217]. Gold nanoboxes have been used as carriers for targeting cancer cells, which were functionalised with folic acid, without affecting healthy tissues and delivering chemotherapeutic agents in A549 cells [292].

Other examples of studies examining NMs-containing products and "Safe by Design" approaches and strategies applied them are a three-tiered "safe-by-design" approach for the eco-benign synthesis of GAPP (poly-cationic peptide functionalized graphene–silver nanocomposite) as a biofilm of Gram-negative bacteria inhibiting and disrupting agent [293], a "safer by design" approach from the company AMIPAINT, applied on its painting products [294], and "Optinanopro", a protocol implementing a "safer by design" approach, and which regards packaging, automotive and solar panel processing lines [http://optinanopro.eu/optinanopro01/files/2018/10/Safe-by-DesignProtocol.pdf].

In general, metal doping, surface coating and covalent functionalization, and adjustment of surface oxidation state and aspect ratio of engineered NMs have been mentioned as the main SbD strategies applicable to NMs [295] [9] [296], with the main techniques being [9]:

- Coating (of which there were mentioned numerous examples above) and encapsulation
- Loading
- Grafting
- Doping

The same review [9] mentions specific principles for safer design of nanomedicines, such as:

- Optimizing the Size and Structure of Nanomaterials
- Regulating Nanomaterial-Related Perturbation of Cellular Redox Equilibrium
- Passivating Defect Sites of Nanomaterials
- Reducing Interaction between Nanomaterials and Biomolecules
- Preventing the Leakage of Toxic Components
- Controlling the Biopersistence of Nanomaterials

- Introducing Stimuli Responsiveness

Of key interest to the project is the concept of safe-by-degradation, which adds to the SbD concept and provides for the optimised life-time of a NM, as well as its safe clearance from the body and the environment [297]. This can be achieved, in principle, using several SbD strategies, with surface modification and functionalisation being key, as it can be structured to include "degradation initiation centres" in the form of specific defects [297].

The existing strategies are being applied or implemented in more complex strategies that include the development of integrated approaches for testing and assessment of nanomaterials [297], a toolbox of biophysical and functional *in vitro* assays for the suitability assessment of nanomaterials in the early stages of vaccine development [298], SIA (Safe Innovation Approach) [280] and AOP (Adverse Outcome Pathways) [281]. Interestingly, there has been the suggestion for the establishment of a European Centre "to meet the needs of industry and other parties concerned with the safe and responsible innovation of nanotechnology, by establishing a one-stop shop for a wide variety of nanosafety related services" [299].

The limitations mentioned regarding the SbD strategies and approaches mentioned above, have mainly to do with the complexity of NMs, regarding their properties and behaviour in complex environments, as well as financial and political issues and industry behaviour. It has been reported that some industries may believe that they are applying or implementing SbD strategies, while it may not be the case. This is especially the case for SMEs and start-ups who may have not the necessary infrastructure, processes, and funds to implement these [300]. Furthermore, substantial bottlenecks exist when consensus is required among the different stakeholders and policy makers for the establishment and implementation of SbD strategies at a political level [301], [302].

As stated earlier, NMs present unique properties, which may change based on the surrounding environment. The NMs properties can be divided in intrinsic and extrinsic. The former are related with the NM's properties that are unique to it and differ from their bulk counterparts, while the latter refer to those that can change based on the surrounding environment, i.e., biological, environmental, and how NMs transform when exposed to these [303] [274] [304] [305]. In fact, the same nanoform may have different results on different biological environments or exposure conditions [9] [306] [295]. Similarly, when dealing with the extrinsic properties of NMs, bio corona (usually formed from proteins) seems to be very important for SbD approaches for NMs [307] [308]. Another challenge for the development of complete SbD strategies is the study and understanding of secondary genotoxicity, which is based on immune cells such as macrophages and neutrophils recruited to clear the tissue from foreign NMs leading to ROS and is currently challenging to predict, due to deficiencies in standard monoculture systems [309].

Considering the difficulties in characterising NMs in complex environments, two ways exist to compensate for the lack of relevant knowledge. These are computational strategies, and read-across and grouping of NMs, which is currently lacking. So far, it has not been possible to achieve the grouping of NMs, as relevant high-quality and regulatory relevant data is missing, to guarantee validated results and meaningful conclusions. This also affects computational approaches, for which while on the rise, reluctance exists for regulatory acceptance and implementation in established strategies [304] [310] [307].

4.2.2 Applicability of SbD strategies from other substances to NMs

According to most of the reviewed literature, existing SbD strategies from other substances, either cannot be applied to NMs, or can be applied, but only after specific modifications [276] [311] [312] [313] [314] [315] [316] [317] [318] [319] [320] [321] [322] [323] [281] [8] [324] [325] [306] [326] [327] [328] [329] [330] [331] [332] [333]. On the other hand, some studies shave reported that existing SbD for other chemicals or non-NMs, are sufficient for NMs, and that SbD strategies and related frameworks made for conventional chemicals, like REACH or by organisations like OECD, are (and can be) applied to NMs as well. [334] [335] [336] [337] [338]

[339] [340] [341] [329] [342].

The bottlenecks for applying such strategies to NMs, are due to their unique physicochemical properties. These include high adsorption capacities, optical properties, increased catalytic activities, which may interfere with the produced results in *in vitro* toxicity assays that can lead to false interpretations and misleading conclusions [313]. Additionally, unusual dosimetry and the agglomeration and aggregation of NMs, are likely to have complex modes of action, like multiple hits at multiple targets, leading to complex threshold–non-threshold dose–response curves, which are difficult to monitor and interpret [314], although this can be compensated by integrative cell-by-cell approaches for genotoxic effects [314]. NMs interact differently with biological environments and macromolecules compared to other chemicals or pharmaceuticals, due to their larger surface area size ratio [315], while extra requirements exist for the exact calculation of the delivered dose that will exert a cellular effect, compared to bulk chemicals, for which, this requirement is not necessary [306].

Furthermore, as stated in the previous section, grouping and read-across of NMs are not currently possible. To achieve these, detailed characterisation, and analysis of a larger number of physicochemical properties are required compared to conventional chemicals [316] [318], which are not usually available, as in most cases only a few physicochemical properties for NMs are reported, like their size and their shape [317]. Nanoreg2 reported main challenges for the grouping of NMs [323]:

1. Limited data availability, which can be mitigated by storing produced data and required metadata in publicly available databases, while the application of quantitative read-across algorithm (nano-QRA) has also been proposed to compensate for the missing data. [331]
2. Lack of harmonised experimental methods for NMs testing**.** Mitigation: stronger and more combined efforts by organizations, like OECD, and suitable update of their testing guidelines [328] [329] [330], although relevant NMs-related updates exist from specific OECD guidelines, that is, adaptation of testing guidelines for chemicals, to more suitable for NMs [327], and even of ISO testing guidelines [333].
3. Serious concerns regarding the data quality. Mitigation: More guidance on quality assessment of produced and curated data.

The complex and unique molecular identity of NMs, which is responsible for their biological effects, also makes it difficult to predict their exposure effects (for example in a workplace) [319]. The FP7 project NanoImpactNet, concluded that EURES (The European Union System for the Evaluation of Substances), a system for the prediction of fate for chemicals, is not sufficient for its application on NMs, because of their unknown rates of dissolution and aggregation [320].

Similarly, the lack of understanding and data regarding the NMs-related MIEs (molecular initiating events) and KEs (Key Events) inhibits the detailed mapping of AOPs in the case of NMs, which are related to SbD strategies and are being used in the case of conventional chemicals [281]. Such evaluation is required and there are ongoing projects working on the matter (e.g., NanoSolveIT) that combine experimental with computational approaches to achieve this. In any case, enough high-quality data are needed to be able to modify, where possible, existing SbD strategies of conventional chemicals and substances for NMs, while computational methods can also assist with filling gaps and guiding the required modifications. Therefore, strong research is ongoing under the remit of standardisation organizations like OECD and ISO, as well as from numerous frameworks and initiatives, towards nanospecific modifications of existing testing guidelines and toxicity and safety assessment for conventional substances.

4.2.3 Applicability of existing SbD strategies to NMs or NMs-containing products

The review of existing literature demonstrated that SbD are being applied to NMs or NMs-containing products, including their risk assessment [336] [304] [338] [341] [326] [328] [310] [343]. Specific examples of such applicability include:

- Substances for topical application, for the consolidation of cultural heritage artifacts, and of coatings already in the industry [344] (application of the Nanoreg "Safe by Design" approach).
- On TiO_2 NMs and multi-walled carbon nanotubes [323] (grouping and read-across, following the workflow proposed by ECHA).
- Products containing carbon nanotubes [345] [346]. Regarding this case, a SbD approach is incorporated into the development of pilot pipelines of production, which is based on risk assessment and tries to minimise the risks of NMs, for example by their reduced emission as airborne particles. This framework for the application of SbD for the aforementioned pilot pipelines, was adopted by EN ISO 12100: 2010 [346].
- In the paint and coating industry products [294] (Application of a "Safer by Design" approach, through a risk/benefit analysis in each life cycle stage of the product).
- On nanomedicines [311] [312] (application of hazard evaluation strategies (HES) and QSAR models).
- On antimicrobial coatings (AMC) [347] (applying SbD techniques like Fe-doping for ZnO NMs, and polymer coating and modification with poly ethylene glycol (PEG) for Ag NMs.)
- On orally administered nanocarriers [348] (SbD approaches based on the understanding of specific signal pathways responsible for cell death after exposure to NMs and use of *in vitro* immunotoxicity tests)
- On polymeric nanobiomaterials for drug delivery [327] (SbD approaches based on the understanding of the toxicokinetics of NMs and their modulation of the immunological system).
- On a poly-cationic peptide functionalised graphene–silver nanocomposite (GAPP) [293] (SbD approach comprised of three tiers: eco-benign synthesis of rGOAg (reduced Graphene Oxide Ag nanocomposite) and functionalisation with poly-cationic peptide (PCP). This approach achieves both enhancement of antibacterial properties and minimization of the toxicological impact)
- On gold nanoboxes [292] (A 3-tiered SbD approach can be applied, Figure 2).
- NMs-containing consumer sprays and powders [340]. (Application of the so called ConsEXPO and ConsEXPO nano models. The first deals with the exposure estimation of NMs via the oral and dermal route, while the latter deals with the inhalation exposure to NMs and was developed by RIVM in 2015).
- On airborne nanoobjects by 3D printers for the manufacture of bone implants [349]. (Application of SbD strategies identified by the EU-project FAST (GA 685825), which relate to the occupational risk by a possible air-borne emission of nano-objects and their agglomerates and aggregates.)

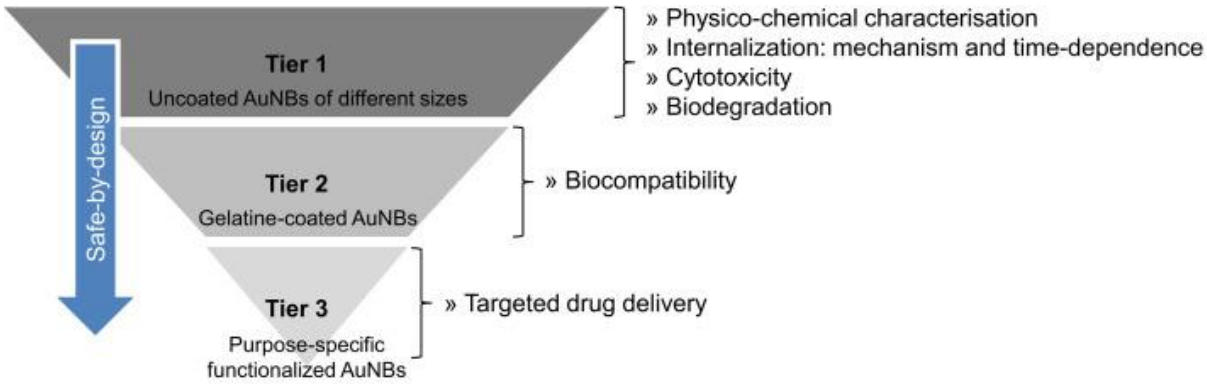

Figure 2. Tiered approach for the SbD of gold nanoboxes.

There are several companies applying the SbD frameworks of NANoREG and Prosafe/Nanoreg2 framework [350]. These companies and their respective SbD strategies are: HIQ-nano, Group Antolin and Nanomakers (SbD to reduce the hazard), Avanzare, Group Antolin, Nanomakers (SbD to reduce the exposure to workers), NanoGap (to reduce the waste and protect the environment), and nanoComposix (SbD to protect consumers). The replacement of complex SbD strategies with less sophisticated and more simplified methods, like method risk reduction through reduced exposure [351] has also been reported.

4.2.4 Modifications for the applicability of traditional SbD approaches to NMs

As stated previously, NMs present much more complicated properties and behaviour compared to bulk and conventional chemicals. This leads to more extensive data requirements from an experimental perspective, and the need for the development of robust and validated computational workflows to fill data gaps, uncover their mechanism of action and predict their behaviour. In this way, it will be possible to analyse the requirements for the update and customisation of existing SbD strategies and especially for their grouping and read-across that exist in the case of conventional chemicals [352] [323] [353] [325] [297] [320] [354] [342] [355]. To achieve this, better data curation, handling and processing workflows are needed, as well as dedicated databases providing access to readily usable datasets [327] [350] [310].

There are already relevant testing guidelines (TG) from organisations like the OECD, regarding toxicity and safety of NMs, that are already applied for conventional chemicals, and according to some of the reviewed papers, are sufficient for NMs as well [356]. But the majority of the methods require modifications for the application of the aforementioned guidelines on NMs, and many of the reviewed papers report such modifications [322] [329] [328] [303] [304] [356] [320] [354] [334] [148] [357]. Specific cases dealing with modifications of such methods, especially in the case of the NMs intrinsic and extrinsic properties and their behaviour in the environment and biological organisms:

- Quick screening of large amounts of nanomaterials by HTS (high throughput screening) techniques, would be useful for more straightforward SAR (structure-activity relationships) [356].
- Need for assessment of the exact effective cellular dose of NMs compared with routine testing of soluble chemicals [306].
- Information on internal concentrations (also referred to as internalised dose) would add value and consequently, would be useful to inform a SbD methods [304]. In general, this dose-response issue is one of the most challenging issues in the determination of the safety and toxicity of NMs.
- Modifications of existing biomarker screens could include the use of phagocytosis and endocytosis-related assays. [358]
- There is a need to modify the Organization for Economic Cooperation and Development (OECD) tests to enhance the concept that removal and uptake into the organism need to be considered as an important part of bioaccumulation and consequently of toxicity [359].
- There should be standardisation and validation of specific *in vitro* assays mimicking defined *in vivo* endpoints [360].
- There is a limited ability of the present *in vitro* assays to deal with secondary toxic mechanisms and organ specificity .
- Understanding in detail the mechanisms underlying inflammasome activation by various NMs may allow for refined "safe-by-design" approaches [361].
- There are needed modifications taking into consideration, challenges related to NM physicochemical properties and their interaction with surrounding and target environments, cellular uptake and novel endpoints, particularly epigenetics [326].

Regarding computational data and models suggested approaches towards more effective SbD approaches include: a) Advanced machine learning methods, like transfer learning [310], b) the

development of more suitable descriptors for QSAR studies [353] [362] c) the use of Structure–activity prediction networks (SAPNets) [363].

4.2.5 Ongoing actions for the development of SbD strategies for NMs (e.g., EU funded projects)

SbD-related actions, projects, and frameworks, for the development of relevant strategies for NMs have a long tradition in Europe and globally. Many of the recovered actions, regard a wider framework of safety and toxicity. Such actions incorporate concepts related to SbD like risk management (RM), risk assessment (RA), hazard assessment, regulatory preparedness (RP), and the safe innovation approach (SIA). The most representative actions funded by EU for the development of SbD strategies for NMs, are the projects NANoREG, Nanoreg2, and Prosafe. These 3 actions are presented in Table 5 below:

Table 5 – The three representative European actions NANoREG, Nanoreg2, and Prosafe, for the development of SbD strategies for NMs.

Action	Description	Period	References
NANoREG (https://publications.jrc.ec.europa.eu/repository/handle/JRC105651)	A common European approach to the regulatory testing of nanomaterials.	Mar. 2013 – Feb. 2017	[364]
Nanoreg2 (https://www.nanosafetycluster.eu/nsc-overview/nsc-structure/steering-group/nanoreg2/)	Development and implementation of grouping and SbD approaches within regulatory frameworks.	Sep. 2015 – Feb. 2019	[364]
Prosafe (https://www.rivm.nl/sites/default/files/2018-11/ProSafe%20White%20Paper%20updated%20version%2020170922.pdf)	Promoting the implementation of Safe By Design	Feb. 2015 – Apr. 2017	[364]

On the following Table 6, a more detailed list of recent, either concluded or still ongoing actions, for the development of SbD strategies for NMs, as well actions related to their safety and toxicity, are presented:

Table 6 – Actions, projects, and approaches for the development of SbD strategies or strongly related concepts, for NMs.

Action	Description	Period	References
ASINA	Sister program of SbD4Nano, which will support the further integration of SIA in EU H2020 projects, facilitated by their collaboration within the EU NanoSafetyCluster		[273]

	working group on Innovation and Safer by Design.		
BIORIMA	A risk management framework for nano-biomaterials (NBMs) used in medical devices (MD) and advanced therapy medicinal products (ATMP).		[365]
CALIBRATE	A project to facilitate risk assessment and management of existing and emerging MNs and nano-enabled products.		[366] [272] [367]
DF4nano Grouping	A decision-making framework for the grouping and testing of nanomaterials.		[323]
EC4SafeNano	Accepted for funding by European Commision	Nov. 2016 – Oct. 2019	[299]
ECETOC			[338]
EU-ToxRisk			[364]
FAST	An EU project, which tries to address the risk to health resulting from the potential air emission of NOAAs by the FAST 3D printer prototype. This project was previously on reported.		[349]
FP7 EU SANOWORK	(Safe Nano Worker Exposure Scenarios) Collaborative Project, which regards exposure of workers to nanomaterials. It is also a project which has attempted to overcome challenges and provide good examples for the demonstration of the SbD proof of concept.		[284]
FP7-eNanoMAPPER	A project which proposes a computational		[367]

	infrastructure for toxicological data management of engineered nanomaterials (ENMs) based on open standards, ontologies and an interoperable design to enable a more effective, integrated approach to European research in nanotechnology.		
FP7-NanoMILE			[364] [368] [367]
FP7-NANOSOLUTIONS			[367] [369]
FutureNanoNeeds		Concluded in 2017	[277]
GoNanoBioMat	SbD approach, which allows identifying and addressing the relevant safety aspects regarding polymeric nanobiomaterials (NBMs).		[364] [272]
Gov4Nano	EU H2020 project which can play an important role in a Safe Innovation Approach.		[273]
GRACIOUS	A framework, which aims to facilitate the application of grouping of nanomaterials or nanoforms (NFs).		[364] [370]
GUIDEnano	A research project that has attempted to overcome challenges and provide good examples for the demonstration of the SbD proof of concept.		[364] [367]
Hazard Evaluation Strategy (HES)	A strategy for injectable nanoparticles, which is a three-tiered concept covering physicochemical characterization, nanoparticle (bio)interactions, and hazard assessment. It is the first hazard		[311]

	evaluation strategy designed for nanotherapeutics.		
iNTeg-Risk	Emerging Risk Management Framework (ERMF), of IRGC for NMs with specific guidelines on governance of emerging risks.		[366]
ITS-NANO	An EU FP7 project, which was one of the first projects for the risk assessment of NMs.	2012-2013	[323]
MARINA	A risk assessment strategy (RAS), which built upon and extended the ITS-NANO approach. It is also a grouping and read-across approach, and regards safe innovation.		[348] [368] [338] [340] [366] [371]
MembraneNanoPart	EU "modelling project", funded under FP7 and working jointly within NanoSafety Cluster as well as by COST MODENA action.		[372]
ModENPTox	EU "modelling project", funded under FP7 and working jointly within NanoSafety Cluster as well as by COST MODENA action.		[372]
MODERN	EU "modelling project", funded under FP7 and working jointly within NanoSafety Cluster as well as by COST MODENA action.		[372]
MODNANOTOX			[356]
NanoFASE			[364][369]
Nanogenotox (http://www.nanogenotox.eu/)			[348] [373] [364]
NanoHouse	A tool and test method being capable of predicting release and transformation of MNs.		[340]

NanoMICEX	Research project that has attempted to overcome challenges and provide good examples for the demonstration of the SbD proof of concept.		[372]
NanoPUZZLES14	EU "modelling project", funded under FP7 and working jointly within NanoSafety Cluster as well as by COST MODENA action.		[372]
NanoRA	A Nanospecific approach to Prioritization and Risk Assessment.		[281]
NANORIGO	EU H2020 project which can play an important role in a Safe Innovation Approach.		[273]
NanoTEST (http://www.nanotest-fp7.eu/)	One of the first projects for risk assessment of nanomaterials.	Concluded in 2012	[277]
NANOTRANSKINETICS			[356]
NanoValid (http://www.nanovalid.eu/)	Safe innovation.		[348] [373] [364]
PATROLS	Developing *in vitro* models, methods and computational tools for MN hazard assessment targeting key events (KEs) in developed Adverse Outcome Pathways (AOPs).		[281]
PLATFORM (H2020, GA 646307)	A project mentioned previously, aiming to develop three new pilot pipe lines (PPLs) for the manufacture of carbon nanotube-based nano-enabled products (buckypapers,treated prepregs, doped veils). The project also aims to reduce the risk of emitted substances (including nanomaterials).		[374] [345]
PreNanoTox	EU "modelling		[372]

	project", funded under FP7 and working jointly within NanoSafety Cluster as well as by COST MODENA action.		
Qnano (http://www.qnano-ri.eu)	A project financed by the European Community Research Infrastructures under the FP7 Capacities Programme.		[284]
QualityNANO	A European project promoting safe innovation.		[348] [335]
RiskGONE	EU H2020 project which can play an important role in a Safe Innovation Approach.		[273]
RIVM/ARCADIS			[338]
SABYDOMA	Sister program of SbD4Nano, which will support the further integration of SIA in EU H2020 projects, facilitated by their collaboration within the EU NanoSafetyCluster working group on Innovation and Safer by Design.		[273]
SAbyNA	Sister program of SbD4Nano, which will support the further integration of SIA in EU H2020 projects, facilitated by their collaboration within the EU NanoSafetyCluster working group on Innovation and Safer by Design.		[273]
SbD4Nano	A new EU H2020 project, aiming to compute an e-infrastructure for the performance testing and implementation of a Safe Innovation Approach in selected nanotechnology supply chains.		[273] [375]
Scaffold	Research project that		[372]

	has attempted to overcome challenges and provide good examples for the demonstration of the SbD proof of concept.		
SCENIHR			[338]
SERENADE	A French project		[376] [273]
SmartNanoTox	A project, which is using results from *in vivo*, *in vitro* and *in silico* research to develop AOPs for adverse pulmonary effects following MN exposure.		[377]
SUN	A research project that has attempted to overcome challenges and provide good examples for the demonstration of the SbD proof of concept.		[340] [366] [371]
SUNDS			[367]

4.2.6 Emerging approaches for the SbD of NMs

The emerging approaches for the SbD of NMs can be divided in two categories: a) New or improved computational approaches, and b) new or improved frameworks for the safety and risk assessment of NMs. Computational approaches include, among others, the development of QSAR or nanoQSAR models. A consistent problem with the development of such workflows is the lack of suitable descriptors and more importantly data for NMs [363] [378] [379] [380]. This is why, a substantial number of studies focuses exactly on the development of improved nanospecific descriptors for the development of more robust QSAR models [381] [378]. The FP7 MODERN, and the H2020 NanoSolveIT and SmartNanoTox projects are examples of these efforts.

In the same framework of emerging computational approaches and improved QSAR models the development of advanced machine learning, deep learning, and artificial intelligence models are included, e.g., GANs (Generative Adversarial Networks) [363] [380] [382] [383]. Such efforts include the prediction of the physicochemical properties of NMs based on physicochemical and atomistic characteristics, like in the case of zeta potential [123]. Again, the biggest problem for the development of successful machine learning models for the prediction of toxicity of NMs, is the lack of sufficient high-quality data [382] and standardised reporting templates, for systematically and transparently describing models that could potentially be used to support regulatory risk assessments of NMs [385].

Another important effort is the approach of Structure–Activity Prediction Networks (SAPNets), which overcome the limitation of finding appropriate descriptors [363], grouping and read-across methods [323], as well as control banding [323]. Regarding grouping and read-across, related projects and frameworks have been the SERANADE project [273], the GRACIOUS framework [370], and NanoRA [386]. NanoRA is a nanospecific approach to Prioritization and Risk Assessment, while the SERANADE project proposes a new, product-oriented approach for SbD [273].

The SIA (Safe Innovation Approach) [375] [280] is a transdisciplinary approach, underpinned

by the One Health concept for nanotechnologies in agriculture [387], an integrative, cell-by-cell approach for genotoxic effect [343], an Adverse Outcome Pathways (AOP) framework for risk assessment [281], and the development and use of Standard Operating Procedures (SOPs) to overcome *in vitro* assay limitations regarding nanocarriers [348]. Additional recovered approaches include the integration of untargeted metabolomics for the hazard/risk assessment of future nanotech products [388], a self-sustained European Centre for service provision in safe and sustainable innovation for nanotechnology [299], a sustainability assessment framework implementing the SbD concept, applied for the first time in the domain of conservation of art works [389], the GoNanoBioMat SbD approach for the development of nanomedicines [272], an integrative approach in a safe by design context combining risk, life cycle and socio-economic assessment for safer and sustainable nanomaterials [279], and a mathematical framing of Multi-Criteria Decision Analysis (MCDA) to illustrate how safety-by-design might be operationalized [390].

Additional data-related approaches for nanosafety are harmonised ontologies and data-sharing practices, high throughput screening to speed up hazard assessment, and new web-based tools for risk assessment of nanomaterials, [367], which can help overcome the problems of data fragmentation and lack of harmonisation. These are in accordance with experimental approaches with SbD applicability. Such e approaches include, among others, the salinization of SiO_2 NPs used as consolidants [391], the use of gemini surfactants, the development of safe and multi-purpose nanostructured lipid carriers in pharmaceutical applications, and the use of nanostructured nickel black surfaces [392]. In general, experimental SbD approaches in nanomedicines include [9]:

- Optimizing the Size and Structure of Nanomaterials
- Regulating Nanomaterial-Related Perturbation of Cellular Redox Equilibrium
- Passivating Defect Sites of Nanomaterials
- Reducing Interaction between Nanomaterials and Biomolecules
- Preventing the Leakage of Toxic Components
- Controlling the Biopersistence of Nanomaterials Introducing Stimuli Responsiveness

4.2.7 Methodologies to demonstrate the safety of NMs. Is full re-evaluation necessary or could demonstration of the reduced exposure / persistence suffice?

The degree of confidence of a certain tool, methodology, or framework used demonstrate the safety of NMs or NMs-containing products rely heavily on the sufficiency and quality of the data used to develop this tool or methodology and the data used for the evaluation. There are conflicting reports in literature regarding the sufficiency of such tools with some claiming that they are [393] [394] [395] [280] [396], while others emphasize the need for more data in terms of volume and quality, and consequently the need of a full re-evaluation [280] [386] [397] [398] [399] [400] [401] [402] [403] [404] [405] [406]. The reason behind this is the increased uncertainty due to the lack of nanospecific information [280], the lack of in depth knowledge of possible adverse effects of NMs for a quantitative risk assessment [386], difficulties on acquiring data and developing standardised and validated analytical methods for NMs exposed in complex environments [397] [404], and lack of detailed information about the life cycle of nanoproducts [398].

Regarding the study and monitoring of reduced exposure or persistence, there have been studies demonstrating reduced (bio)persistence of digested NMs [407] and inhaled NMs [342]. Specific available tools and frameworks that manufacturers could take advantage of include:

- mechanistic toxicity screens and control banding tools [396] [400]
- the Nano Risk Framework, the Precautionary Matrix, and the Stoffenmanager nano from Netherlands [408] [275] [375]
- the methodology of the EU FP-7 GUIDEnano project [409]

- NANO LCRA, a proposed life cycle risk assessment framework, regarding cellulose nanomaterials [410]
- the 'NANoREG Toolbox' [76]
- the web-based SUN Decision Support System (SUNDS) [411]
- databases for engineered nanomaterials (ENM) toxicity assessment such as eNanoMapper, NanoMaterialRegistry, and Nanoparticle Information Library [412]
- Horizon scanning (HS) [366]
- The GRACIOUS framework
- A methodology of the Quantitative Risk Assessment (QRA) [413]
- A flow cytometry-based micronucleus scoring for reliable nanomaterial genotoxicity assessment [414]

As reported in sections 4.3 and 4.4, tools developed for conventional chemicals can be applied on NMs usually following modifications to account for the unique character of NMs. In this case, some re-evaluation would be needed based on the updated procedures. This may include bar coding NMs to track their fate in the environment and accept responsibility in case of an accident or adverse effects [275]. While this is not a methodology to prove that a NM or NM-containing product is safer, it can assist with higher transparency, responsibility, and care, towards society and the environment and provide data regarding a NMs lifecycle.

4.2.8 Can SbD strategies and their public communication help with promoting the societal acceptance of NMs-containing products

Safety is an important public value [415] and therefore a prerequisite for the societal acceptance not only of NMs-containing products, but also for every kind of new and innovative product or technology. While it is not feasible to prove that a product or a technology is 100% safe, but just safer [416], transparency is key for societal acceptance. This means that the concepts of "safe by design" or "safer by design" strategies can promote societal acceptance of NMs-containing products if used correctly [417] [418] [419] [420] [421] [300] [422] [312].

In fact, public demand for safer and functional novel products and technologies has pushed researchers to think about and deliver "safe by design" or "safer by design" strategies [420]. This means that transparency and public communication, on the side of manufacturers, retailers, and regulatory bodies of the risks, benefits, and of course, safety and SbD strategies for NMs, and NMs-containing products are key for promoting acceptance and sustainable growth. Unclear communication not only to society, but also among the engaged stakeholders, is in many cases a significant obstacle to making NMs and NMs-containing products more acceptable [312] [421] [419] [423] [313].

It is also worth mentioning, that the tendency of not publishing studies with no demonstrated adverse effects seems to also lead to decreased public trust [424], which conflicts the established scientific publication practices. Thus, the publication of only negative/toxic, which in many cases are misinterpreted and communicated wrongly to the wider public may lead to the conclusion that NMs and/or NMs-containing products are inherently unsafe. The publication of plain language summaries to explain the underlying concepts in layman wording and/or a change in mindset towards publishing more studies with no demonstratable effect can offset such a risk.

To this end, a series of tools and frameworks that aim to facilitate the communication between stakeholders and the society, and among all the different stakeholders in general have been developed and can be used to increase transparency, public trust, and acceptance:

- the concept of "Trusted Environments" [423] [425] [419].
- the concept of a "*SbD Scenario"*, which is defined as a combination of safety element(s) and a level of ambition on a specific safety element [280].

- the EU-funded GoNano project, which has provided guidelines, to address the reluctance of various customers and companies to use nanomaterials due to lack of knowledge on their safety and regulations [375].
- the LICARA nanoSCAN project, regarding antimicrobial coatings in healthcare settings [313].
- combining multi-omics tools at one platform, which can lead to acceptance of nanotechnology products from the general public [388].
- the GUIDEnano tool, which can promote risk communication to regulators, insurance companies, and society.

4.3 Experts survey results

The surveys were shared via email with a total of around 100 experts relevant to either the topic of (bio)degradation of NMs and organic coatings or the SbD of NMs. Furthermore, the surveys were shared with the OECD and the respective Working Group on Manufactured Nanomaterials (WPMN) and other relevant experts to capture the opinions of the OECD related experts and the current state-of-the-art and ongoing work within the organisation. The responses received via communication with the OECD are presented separately in each subsection, while an overall conclusion is offered.

4.3.1 (Bio)degradation of NMs and organic coatings

For the (bio)degradation of NMs and organic coatings, a wide range of materials was included as studied by the experts that submitted responses. From the range of responses, it is evident that the focus of research has shifted to state-of-the-art novel and advanced NMs. Such materials have several functionalities in the fields of nanomedicine, personal care, energy etc. For example, hexagonal Boron Nitride (hBN) has excellent thermal and chemical stability and respective properties and has been used in high-temperature equipment, laser printers, cosmetics and more [426], [427]. These included:

- Graphene based materials
- Carbon based materials (functionalised or not)
- 2D materials (functionalised or not, e.g., MoS_2, hBN)
- Micro/nanoplastics
- Nanocomposites (carbon or 2D materials in polymeric matrices)
- Fullerenes
- Metals
- Metal oxides

The first section of the questionnaire dealt with the potential definition of NMs degradation and (bio)degradation. The experts were asked to express their opinion on whether a common definition of these was plausible, and whether it could be applicable to organic coatings as well. Most experts agreed that a common definition for NMs degradation and (bio)degradation was possible, as the difference between the two was in terms of the release medium and the process itself. This meant that the same techniques would be used for studying and monitoring the process. Furthermore, the experts agreed that these would be applicable to organic coatings as well. Some objection was expressed for the potential of having a universal NMs (bio)degradation definition, which was based on potential different mechanisms of action for degradation and

biodegradation. This is also mirrored in the techniques that have been proposed for monitoring the two processes, as degradation is mainly linked to dissolution, while biodegradation to enzymatic/aerobic degradation.

Regarding the proposed definitions, these were focussed on the specific transformations that may take place in different exposure environments. These were either in terms of their physicochemical properties (e.g., shape, size, crystallinity, surface properties) over time and compared to the starting material, i.e., the stable version post-synthesis. Another approach was that of chemical transformation of the materials, which transforms their oxidation state and leads to by-products. These in the case of carbon-based NMs could be CO_2 and in the case of metals or doped carbon-based NMs other metal oxides, or the formation of molecular ions for the precipitation of other NMs and substances not in the nanoscale (e.g., decomposition of carbon nanotubes, graphene, and fullerenes to other structures of C). One submission defined (bio)degradation as the process that can transform materials under natural or biological environments to non-toxic products, although the validity of such remark is debatable.

In general, the NMs biodegradation has been suggested to be defined in the same way as degradation, with the caveat that it takes place in biological environments. It has been pointed out, though, that it should be defined as decomposition in the normal conditions of the environment (those environmental compartments to which the nanomaterial is expected to arrive). It was suggested, as well, the biodegradation should not be confused with biodegradability. The former is not the possibility that NMs can biodegrade, but the quantitative certainty that they do in the usual environmental conditions of light, temperature, humidity, bacterial and other microorganism presence. The current study focusses on the actual biodegradation process and how this can be monitored and studied. Furthermore, biodegradation should be separated from biotransformation, which can be considered a broader "umbrella" term for various types of transformation that may occur in different systems and in biological systems and reserve "biodegradation" to biological (e.g., enzymatic) mechanisms as opposed to other forms of degradation which may be chemical (dissolution) or physical (UV light). Additional forms of biotransformation including not only dissolution but also recrystallization - as well as "bio-corona" formation should be considered as well.

ECHA's guidance on degradation distinguishes between the "biotic" and "abiotic" degradation processes, while defining biodegradation as that performed by microorganisms. Recent knowledge, though, demonstrates that enzymatic degradative reactions may also occur in mammalian systems through the actions of peroxidases (e.g., neutrophil myeloperoxidase, MPO) or through a peroxynitrite-driven reaction which in turn depends on the joint activities of two enzymes namely the NADPH oxidase and the inducible nitric oxide synthase (iNOS). Thus, it was suggested that the definition of biodegradation should encompass all types of enzymatic degradation, irrespective of the origin. Other forms of "biotic" degradation should be considered as well, e.g., degradation in cells, which are not necessarily enzymatic. Such examples may include the dissolution of NMs or their coatings in the acidic environment of lysosomes, which is the final "destination" of most nanomaterials following their uptake by cells.

Following the definition of (bio)degradation, the respondents were invited to comment on the techniques that can be used for the studying and monitoring of (bio)degradation. A wide range of techniques was reported, which agree with those identified during the literature search. These included:

- Dissolution studies
- Aerobic degradation:
    - Measurement of Biological Aerobic Degradation of the organic coating in controlled closed environment
- Enzymatic degradation – "enzymatic attack"

- Microorganism degradation
- Photo-Fenton reaction
- Oxidative conditions
- Raman spectroscopy combined with (TEM) imaging for carbon based and 2D materials
- Optical microscopy
- AFM
- TEM/TEM liquid cell with chemical mapping
- SEM
- STEM
- DLS
- To track degradation of metal NMs: ICPMS/ICPMS imaging
- XPS
- XANES
- UV-Vis
- Fluorescence spectroscopy
- Gel electrophoresis
- MAS-NMR
- Hydrolysis
- Carbon dioxide formation
- Oxygen demand
- Organic carbon loss
- EDX

For the applicability of traditional monitoring methods, the experts believed that they are applicable or are applicable with modifications. While no specific modifications were proposed, it was mentioned that these should be dedicated to NMs characterization and would need to assess the loss of the nanoform structure (without losing the chemical composition, e.g., $AgNO_3$ nanoparticles that could aggregate to bigger particles that are not considered NMs anymore, but which chemical composition maintained unalterable).

For existing regulations, most experts mentioned OECD guidelines. However, this related to just two existing guidelines, namely TG301 and TG316, which were also identified during the literature search and the ENV/JM/MONO(2018)11 OECD - Assessment of Biodurability of Nanomaterials and their Surface ligands in Series on the Safety of Manufactured Nanomaterials. Other guidelines mentioned were the ISO standard "ISO 10993-9:2019" that applies to the degradation of medical devices in the body (excluding purely mechanical degradation). Specific

modifications required for traditional guidelines to be applicable to NMs, were tests for monitoring the oxidation state of the elements composing the NM and the improvement of the resolution for imaging-based techniques to be able to image very small NMs sizes. In general, the focus should be on technological progress of the methods than modifying the methods themselves.

Finally, the opinion was expressed that data are lacking regarding the (bio)degradation of NMs and organic coatings. This is especially true for novel and advanced NMs, as "traditional" NMs like SiO_2, TiO_2, $AgNO_3$ have been extensively studied. It was the experts' opinion that public funding is required to push forward, study the NMs (bio)degradation and fill that gap with high-quality data. To achieve this, new, simpler validated methods and protocols are required to monitor and study NMs (bio)degradation, which will also depend on the type of NM as it will be difficult to identify a common method for all. These methods should work in abiotic settings, able to monitor the dissolution of NMs that may not be possible with traditional methodologies. Another challenge would be to study the degradation of a NM and its coating simultaneously. In any case, these methods will be dependent on the combination of various techniques. This will also mean that existing methods and approaches which can be used to study chemicals (bio)degradation, will need to be evaluated for their applicability to NMs and enhance their resolution so real-time and *in situ* measurements is possible especially in the case of organic coatings (due to their thin layers). The outcome of the produced/exploited methods will need to be able to identify and fill gaps around the specific by-products following (bio)degradation, i.e., their identity, physicochemical characteristics, fate in the environment or organisms, and their adverse or beneficial effects. Thus, research should focus on these topics, studying fast- and slow-degrading NMs and organic coatings employing short- and long-term studies with enhanced resolution and capabilities.

4.3.1.1 (Bio)degradation of NMs and organic coatings – OECD responses

In total, 9 responses were received via the OECD from experts studying the (bio)degradation of NMs and organic coatings. The experts reported that the NMs with which they are working with included:

- Metal oxide NMs (TiO_2, SiO_2, $K_2O{\cdot}nTiO_2$),
- Carbon nanotubes (DWCNTs, MWCNTs).

NMs degradation was defined by the OECD experts, following collation of the received responses, as any biological, physical, or chemical process (e.g., ageing, weathering, oxidation, UV degradation) that results in a significant modification to the core atoms of a material with nanoscale properties via the loss of atoms, ions, or molecules. Most experts (78%) agreed that the degradation of NMs includes their organic coating, and that the definition could apply to both. The rest of the experts believed that the degradation of organic coatings (coatings including proteins, lipids) takes place *in vivo* when phagocytized by macrophages or neutrophils or segregated or covered with granuloma. In these cases, some experts mentioned that the formation of protein corona on NMs should be considered as an organic coating as well.

Like the NMs and organic coatings degradation, their biodegradation was defined as any biological process or biologically mediated process (e.g., interaction with biota leading to bioaccumulation, degradation by enzymes) that results in a significant modification to the surface or core atoms of a material with nanoscale properties via the loss of atoms or molecules, and ideally lead to the NMs full disappearance. For biodegradation, all experts agreed that the specific definition covers both the NMs and organic coatings. It was also suggested that the biodegradation of organic coatings should consider bare (uncoated) NMs covered in natural organic matter following release into the environment.

Thirty three percent (33%) of the OECD respondents believed that the definitions of NMs degradation and biodegradation should be the same. Some of them expressed the opinion that

a common definition for NMs degradation and biodegradation is possible e.g., NMs coated with organics could easily undergo phagocytosis, pinocytosis or gill filaments work resulting in the bioaccumulation in biota. These NMs could adsorb to any organics in the environment, which took a long time to release back to the environments. Two respondents suggested that a common definition should mainly include the NMs ionisation. The experts that did not agree with a common (bio)degradation definition proposed instead the grouping of NMs based on the NMs nature (e.g., organic vs. inorganic), applicability of different degradation mechanisms and their efficiency, groups based on the degradability of metal-based nanomaterials in water, and grouping defined under the Gracious Framework [370], [428]. Finally, one of the experts suggested that (bio)degradation definition is common for nano and bulk materials and that should be mirrored to the techniques and SOPs used for their study.

The proposed specific approaches for the study of NMs (bio)degradation agreed with those identified during the ELS and the project's surveys in general. Same as before, enzymatic attack, cellular uptake, citric acid cycle, acidic by-products production, and oxidation were flagged as the key processes for studying (bio)degradation. These were complemented with NMs characterisation techniques like TEM microscopy, the single-particle ICM-MS, and TOF-MS. Techniques to evaluate the coating, e.g., TGA can be used for the study of (bio)degradation as well. To monitor the NMs (bio)degradation, Raman spectroscopy combined with TEM or Field Flow Fractionation could be used, whereas for the monitoring of the (bio)degradation of organic coatings no techniques were mentioned. In agreement with the other surveys, most experts agreed that techniques used to study or monitor the (bio)degradation of bulk substances cannot be used or need substantial modifications to be applicable to NMs and/or organic coatings. These modifications include analytical and resolution improvement and the development of NMs-specific SOPs, in agreement with the non-OECD received responses. The rest (33%) mentioned that the bulk techniques could be used as it is.

Considering the standardised techniques and regulatory frameworks for the study of the (bio)degradation of NMs and organic coatings, one respondent suggested that these should be the same as those used for bulk substances. However, another expert suggested that modifications should be performed to the existing standardised techniques e.g., to the OECD TG 301. These modifications, in agreement with the suggested techniques, focussed on improving the analytical quantification of the test substance (especially in real life exposure scenarios and concentrations) to be applicable to NMs. Additionally, any aspects of the test that may complicate quantification should be considered (such as test/exposure media).

Regarding the regulatory guidelines, it was mentioned that the Framework for the Risk Assessment of Manufactured Nanomaterials under the Canadian Environmental Protection Act, 1999 [429] describes potential degradation of metal-based nanomaterials that may be considered in fate assessment and in the study design of toxicity testing. The framework, which is currently under revision to better consider NMs dissolution and dissociation, acknowledges that environmental fate models often do not consider kinetics of degradation of nanomaterials, so methods for identifying degradable substances, such as chemical structure need to be identified. Additionally, certain organic NMs, polymers, and cellulosic NMs have been identified as categories "which tend to undergo degradation in the environment. Their degradation products may be further assessed for their environmental fate and persistence."

Finally, the experts commented on the current research gaps regarding the (bio)degradation of NMs and organic coatings and, thus, where research attempts should focus.

- How NMs with/without organics behave under varying exposure scenarios including wastewater treatment plant, release to the surface water or near a stream or river, air, or incineration as well as open field release.
- The lack of standardisation and characterisation techniques for the surface chemistry at environmentally and biological relevant conditions and disposal protocols for NMs.

- The experimental data dispersion (many scattered experiments) for the organic coatings (bio)degradation.
- The lack of studies comparing the (bio)degradation mechanisms of NMs and organic coatings with bulk substances.
- The lack of data on the (bio)degradation rates for composite NMs.

The experts highlighted that future research, which should be supported by public funding, should address the above gaps but also the following aspects:

- Focus on experiments' reproducibility and data analysis.
- Definition of physicochemical properties that are important for determining if standard techniques are applicable to certain types of nanomaterials or not.
- Development of techniques to detect nanomaterials in their actual state in cells and tissues.
- Definition of the generic exposure scenarios mimicking the different biological environments.
- Studies on whether NMs retain their inherent properties following any degradation and transformation processes.

4.3.2 Safe by design of NMs

Same as in the case of (bio)degradation, a wide range of NMs has been reported to be studied by the contacted experts. These include:

- Multicomponent NMs
- High-aspect ratio NMs
- Carbon-based nanomaterials (carbon nanotubes and graphene related materials)
- 2D materials (hBN and MoS_2)
- Metals Ag NMs (including uncoated for applying to wound dressings)
- Metal oxides (e.g., ZnO, SiO_2)
- Magnetic NMs
- Core-shell polymer NMs
- Nanoliposomes

Safe by design was defined by most experts (here a collated definition is presented) as the implementation as a process, which starts at the very beginning of the NMs, nanoforms, and nano-enabled products design and production phase, including the raw materials used. It aims to predict, reduce, and eliminate intrinsic risks (i.e., exposure, release) during their production and use steps, and during the entirety of the NMs lifecycle (from R&D stage to recycling and disposal), without losing the desired functionality. This can be achieved by modifying their physicochemical properties (defined as safe by design) and by establishing safe production processes (safe by process design). It has also been defined as a feedback-loop of modelling techniques to improve the safety of NMs at various scales.

While there was agreement regarding the background of defining this definition, some of the experts divided this into 2 processes:

- Safety-by-Material-Design: The set of physicochemical attributes that can be modified in a given nanomaterial, to produce a biologically more benign version.

- Safety-by-Process-Design: The set of process modifications that can be applied to result in a safer nanomaterial process.

It has been also mentioned that the process should provide a sufficient understanding of the interaction of pristine NMs with corona and how this modulates biological and commercially relevant properties of NMs. This knowledge will allow "rational" design of NMs with predictable and low toxicity, but robust and useful commercial properties. On the other hand, if the predicted or identified risks are not acceptable producers should stop the innovation, re-design and evaluate the risk again or carry out more tests if the uncertainty on the risk is too high.

Some disagreement existed regarding the potential of a universal definition of SbD for NMs. Most experts thought that a common definition is or could be possible. These referred to the same definitions, which were given in the previous question as an example. It was mentioned that a safety-by-process design is highly similar to conventional safety, therefore SbD should primarily be focused on safety-by-material-design. Having said that most experts believed SbD for NMs does not currently exist fully. This was because there is a significant gap in high-quality data available to allow for the development of predictive models that can drive and enhance SbD in the NMs design phase. It was stated, though, that there are sectors, e.g., nanomedicine, which may have a far better track record of SbD. Another example of SbD mentioned, was the use of coatings to reduce biological effects of side effects in nanomedicines, although there is the question of the potential of (bio)degradation of both the coating and NM over time. However, the facilitation of (bio)degradation was stated in one case as a technique of NMs SbD. Similarly, it has been stated that there are companies which are currently aware of the importance of the SbD strategies and have implemented or are trying to implement these during NMs production (e.g., the Stage Gate Model is mentioned in one of the submissions which is also one of the pillars of NanoReg's SbD). These efforts focus mainly on minimising occupational exposure, or by producing products in safer-to-handle forms such as a "paste" form or water-based dispersions as opposed to powders. In general, though SbD for NMs is continuously being implemented into NMs design and production, with examples including the implementation of existing data in early innovation processes, which is already possible and has been demonstrated in certain projects as case studies (e.g., NanoReg2). Other industrial areas have also implemented certain strategies which can be considered to align with SbD, e.g., the tire industry which has established so called trusted environments where safety of tires is discussed across companies and including regulators.

To develop a common definition of SbD for NMs, a set of pragmatic criteria need to be outlined. These may include lists of materials of concern, as described by the recent proposal for risk governance of advanced materials, described in the Joint perspective by the German higher federal authorities. Furthermore, distinction between criteria for SbD of nanomaterials per se and SbD of nano-enabled products may need to be formulated. The former may need stronger focus on material modelling where functionality and safety are modelled in parallel, while the latter may be more strongly coupled to high-throughput screening approaches aimed at ranking and prioritisation of materials suitable to take forward during innovation (or prioritisation for further toxicity testing before taking the materials further).

Currently, a definition of SbD for NMs is being developed within the OECD Steering Group for Safe Innovation Approach (SG-SIA). The concept also covers sustainability today and is referred to as Safe and Sustainable by Design (SSbD). Both the safety aspect and the sustainability aspect are being defined in terms of criteria for each. The basis for the development of the safety criteria stems from work done in e.g., NANoREG, NanoReg2 and Gov4Nano EU-funded projects, and described in detail in in the recent OECD report on the SIA approach. This is also one of the

main ongoing actions for SbD for NMs, which means that the concept is very wide and there are currently no boundaries, as the definitions and criteria of each component (SbD and SSbD, Regulatory Preparedness, Trusted Environments) are still being developed. Other mentioned actions include safety assessment approaches/tools such as ECETOC TRA, Stoffenmanager Nano and ISO/TS 12901-2:2014, EU-funded partnership PARC, and ISO/CEN-activities, definition of SbD (CEN TC352). ISO TR 12885 and ISO TS 12901-2 also exist, however most information included regard safer-by-process design, which is akin to conventional process safety (engineering controls, PPE etc.).

There are several ongoing EU-funded projects (e.g., ASINA, SAbyNA, SABYDOMA, HARMLESS, SUNSHINE) that are working on the SbD for NMs topic, while DIAGONAL focusses on the multi-component and high aspect ratio nano and advanced materials. It has been pointed out that these frameworks are difficult to implement in real case studies. For this reason, it is important to define the purposes of the SbD of the investigated NMs considering their specific applications (e.g., in building materials, medical products, cosmetics or food packaging) and then create a framework with considerations on the specific use of the investigated NMs. Other boundaries include the loss of NM functionality, as well as a lack of understanding of the precise mechanism of action of a NM, which is usually due to several factors including contaminations from catalysers during production, e.g., Ni. As a result, several modifications are required to produce a "safe" NF. Again, it has been pointed out that the lack of sufficient quality data with a broad domain of chemical and physicochemical space, and perhaps the molecular and atomic level hinder the development of SbD, and the development of *in silico* approaches that can help with predicting the properties and behaviour of NMs at the design stage. In general, the lack of data was the key topic mentioned by experts.

Traditional SbD methods for bulk chemicals are believed to be applicable to NMs, with or without modifications. Nonetheless, as NMs do not behave similarly to their bulk counterparts, specifical modifications mentioned were life-cycle analysis tools, but also many types of toxicity testing approaches, which have been subject to modifications to be applicable to NMs [430], [431]. In addition, existing data is a strong player in SbD approaches and the FAIRness of nanomaterial-associated data is crucial [432]. Of course, any modifications will need to consider the physicochemical characteristics of NMs and their transformations in the different biological/environmental systems considered. A specific example mentioned is Hierarchy of Controls, which places substitution after elimination in terms of efficiency in reducing the risk. This could be a bit more detailed in NMs, e.g., having an additional step of substitution with a safer version of the same NM.

This is related with the fact that standardised techniques/methods for the SbD of NMs do not currently exist, although it has been mentioned that many of the existing regulatory guidelines for nanomaterials (e.g., under OECD ENV/JM/MONO(2020)36/REV1, REACH or CLP) can be applied in SbD approaches [433], [434]. Existing techniques for assessing the potential (eco)toxicity of NMs are considered to be reliable – even in complex environments - if if nano-related specificities are considered (e.g., aggregation, agglomeration) and a full characterisation is given. . In such a case, it is possible that no other modifications may be required. The use or development of such processes is generally believed to lead to the re-evaluation of NMs, including respective guidelines, although this may be very sector specific.

Considering the gaps and future steps of SbD for NMs, the key topics are data, the difference between the designed and the actual nanoproducts, the criteria to be used for the evaluation and a general cultural change towards this path. Specific focus should be on these topics and research on New Approach Methodologies, including high-throughput screening, omics and *in silico* modelling, focussing on complex nanomaterials and on nanospecific descriptors, of both physicochemical parameters but importantly also biological data. Special endorsement should be given to the communication among stakeholders to share more knowledge about NMs risks. Other topics include determining the difference on exposure and specially toxicity of the NMs regarding the 'classical' materials since their size, form, and shape can exert higher adverse effects on the exposed living organisms. It was mentioned that the hotspot remains to be mainly

the occupational exposure. There are quite efficient methodologies to provide on-site measurements for exposure to NMs and further expansion on this research area will be beneficial.

In any case, this should be supported by public funding and official guidance on the strategies and their implementation, probably customised for specific groups of NMs and application, which is already challenging. In any case, SbD should be part of the regulatory requirements and as soon as there exists a set of concise, robust, and proven SbD criteria for a given NM, it should be a regulatory requirement to conform with these criteria but should not substitute regulatory evaluation. Finally, there was wide agreement that public communication of SbD strategies will be able to help with the societal acceptance of NMs-containing products, in agreement with the literature search.

4.3.2.1 Safe by design of NMs – OECD responses

In total, six responses were received via the OECD from experts dealing with the SbD of NMs. The experts reported that the NMs with which they are working with included:

- Carbon-based NMs (including carbon nanotubes – CNTs),
- Metal oxides (e.g., TiO_2, SiO_2),
- Metal alloys with organic NMs,
- Complex multi-component, hybrid nanomaterials – MCNMs and,
- High Aspect Ratio Nanoparticles – HARNs.

In agreement with the other responses and the ELS on the SbD of NMs, the proposed (collated) definition of the SbD of NMs as per the responses by the OECD experts is the consideration of the NMs safety issues for humans and the environment along their entire life cycle (manufacturing, processing, use, disposal, and recycling). This definition is like that of bulk substances, and it was suggested that a common definition should be used for both and extended to the concept of SSbD. This definition is similar to that of the FP7 NanoReg project, mentioned in the responses as well, which considered SbD as a process enabling – at early stages – considerations of health and environmental safety in addition to retaining functionality during the design of a material, product, or process.

Consensus did not exist regarding a universal SbD definition. Some experts believe that a common definition is not possible, or that it is difficult to achieve, due to the different positions regarding the definition of "safe"/ "safer" and their respective limits. It was proposed that smart NMs (advanced NMs that actively respond to external stimuli) could be possibly grouped together under a common SbD definition. On the other hand, it was stated that SbD is not so much applicable to groups of NMs as it is a set of steps developers of any products (not just NMs) can take to ensure that safety is considered from the beginning of the material's / product's development.

The applicability of SbD in industry, currently, is applied at a research level during NMs production, e.g., screening of toxicity and exposure during R&D phase to define possible risks and if necessary, alter the product to reduce this risk. This includes, for example, substituting synthetic nano-polymers with nano-cellulose, and more generally the use of safer and renewable starting materials, reagents, and solvents during the NMs synthesis processes. However, on industrial level, it was stated that the NMs SbD depends on the manufacturer's position regarding safety aspects, budget, and possible alternatives, which agrees with the previous responses and the ELS presented in this report. This was complemented with the unwillingness, in some cases, of manufacturers (either NMs or nanoproducts) to invest in safety when it is not known whether their products will be profitable when they reach the market.

Regarding the approaches/strategies used for the NMs SbD OECD and EU projects (e.g., HARMLESS, NanoReg and NanoReg2, proSafe, SUNSHINE, and SbD4Nano) that have been highlighted in this report previously were reported. The REACH regulation was also pointed out as a regulatory guideline that can be used for the NMs SbD, without the SbD substituting the regulatory evaluation. This means that integrating and applying SbD principles into the regulatory process, especially when these are based on the modification of strategies applicable initially to bulk materials, a partial re-evaluation under a regulatory context is potentially required. This agrees with 67% of the respondents via the OECD believing that bulk techniques cannot be used (or if yes, with modifications) for NMs SbD, mainly because NMs act as a suspension, where bulk chemicals dissolve.

Regarding the specific techniques used in NMs SbD, the coating, the facilitation of (bio)degradation, and the stage gate model were mentioned. Considering the gaps and future steps of SbD for NMs, the lack of comprehensive data about the performance, hazard, exposure, and release potential of the great variety of nano-enabled products in use are the key issues that need to be addressed. This is a challenging barrier because there is a substantial difference between actual and designed products, and when a product is in the R&D phase it is difficult to estimate possible exposure (use) scenarios. To fill these gaps and support the NMs SbD, future research should focus on complex NMs, on the creation of new exposure-driven modelling frameworks to reduce toxicity, and to the development of standardised techniques and/or strategies for the NMs SbD (e.g., development of a screening test method as a first step in the SbD process). The interaction with social sciences and the better information (e.g., gaining and sharing more knowledge about NMs risks) were highlighted as future research goals supported by public funding. Finally, the majority (83%) of the experts agreed that the public communication on SbD is required for the societal acceptance of NMs-containing products.

5. Discussion

The current report presents the results of an extensive literature search for the (bio)degradation and persistence of NMs and organic coatings and the SbD of NMs. This is complemented with surveys with experts from both fields and a targeted survey on relevant OECD experts. During the surveys, the participants were asked specific questions with respect to the definition of (bio)degradation of NMs and organic coatings and the SbD of nanomaterials. The aim of the literature study and the surveys was to identify the state of the art regarding the ongoing research, the existing gaps, as well as the outlook in terms of future development and how these can be potentially implemented under a regulatory context.

The results of the ELS study demonstrated that a lot of work is taking place in the field of (bio)degradation [18]–[20], focussing mainly on carbon-based and organic NMs, many of which are more easily degraded compared to inorganic NMs, although certain organic NMs like PFAS are very persistent as well. In fact, many organic NMs (e.g., biopolymers, lipids) are the easiest to degrade, as carbon-based NMs are more persistent *in vivo* [9]. This work is based mainly on microbial, cell and enzymatic degradation [18], [21], with a wide range used for the purpose, e.g., *Pseudomonas* [22], *Trabusiella guamensis* [23], intracellular macrophage degradation [30]–[32], human blood plasma [33], Lactoperoxidase [34], [35], and Horseradish peroxidase (HRP) [19], [20], [35]–[39]. Other forms of degradation studied have been photodegradation and chemical degradation [5], [48], [49]. Photodegradation relies on the interaction of NMs with light (mainly ultraviolet [48], [50]), while chemical degradation on the interaction with chemical reagents [5]. Less work has been identified in the case of organic (polymeric) coatings and is mainly focussed in the field of nanomedicine, where organic coatings are used to stabilise the NM and control drug release and/or penetration into specific tissues and cellular uptake [60]–[62] [63], [64]. Metals on the other hand, have not been extensively studied, as they cannot biodegrade, although the dissolution and speciation of metals may be affected by abiotic and biological interactions yielding changes in their environmental availability [65]. Having said that, some research on the field exists for therapeutic purposes [70] [71].

For the study of (bio)degradation of NMs and organic coatings a wide range of techniques have been used (see section 4.1.5), some of which have been mentioned during the experts' surveys as well. As expected, due to the wider range of the ELS, a lot more techniques have been identified. There is agreement, though, that enzymatic degradation is the most studied method of degradation. This means that a potential research gap exists in the study of cell and bacterial degradation, especially in characterising intracellular transformations (e.g., intracellular dissolution of Ag NPs) which is necessary to assess bioavailability. A key issue in this case, is the need for using and combining state of the art analytical techniques like synchrotron-type experiments. In fact, one of the key gaps identified by the surveys was the need of new validated methodologies for the study of (bio)degradation of NMs and organic coatings, based on the combination of existing experimental techniques. It was stated that these techniques should be simpler, although considering the complexity of the topic this may not be possible. Considering the difference in properties and behaviour of the different types of NMs, such workflows would most likely need to be type-specific, work in abiotic settings, and able to monitor the dissolution of NMs that may not be possible with traditional methodologies.

This principle applies to the simultaneous study of the degradation of a NM and its organic coating and the potential transformations taking place and by products forming. The combination of existing methods and techniques, which may be applicable to traditional chemicals, will need to be evaluated for NMs and organic coatings. The purpose should be their applicability to NMs, allow the retrieval of materials from complex matrices (e.g., environmental, biological), and enhance their resolution during real-time and *in situ* monitoring. This is especially applicable to organic coatings as the degradation timescale would be far shorter due to the thinness of the material.

The overall purpose should be the production of high-quality degradation data, which is a long-standing gap, on both - the mechanism of the (bio)degradation purpose, which remains ambiguous - as well as the generated by-products and potential fate and effects (beneficial or adverse), and in general during the entire lifecycle of the material. Eventually, these data can also assist with the development of predictive models that can assist with predicting the mid- to long-term behaviour and effects from the (bio)degradation of organic coatings, minimising the need for the currently lacking long-term *in vivo* data, which limit R&D capacity to evaluate candidate NMs for clinical and other uses.

Similarly, a lot of work is being performed for the SbD of NMs. In this case, lack of consensus exists on how to define SbD, which is based on the vagueness and broadness of the term [269] and due to the unique nature of NMs and the different properties and requirements for the different NMs types. This means, as also expressed in the surveys, that a more case-by-case approach is required, which can be either NM-type or sector-specific [269]–[271]. As stated in section 4.2.1 the early attempts of SbD focussed on 3 pillars of safer by design [270]:

i) safe products by design are based on the chemical and physical properties of the NMs, such as shape, aspect ratio, crystallographic faces, and chemical additives in nano-enabled products which can maximise benefits and minimise risks
ii) safe use of NMs-containing products, including minimising waste production and safe handling and recycling
iii) safe industrial production to ensure a safe working environment as this is the most likely environment for human exposure to take place.

SbD, with S corresponding to safe and not safer, is considered to be unrealistic since it would not be possible to achieve absolute safety under all possible conditions [269] [277]. This means that research should focus on developing new NMs and nano-containing products that minimise as much as possible any potential risks. Such approaches can be based on established principles that include [435]:

- Design for safety: identify particularly high-risk areas and to minimise both probabilities of occurrence and effects, with systems being analysed through all phases of their life cycle.
- Green chemistry: development of products with lower environmental risk, including the inclusion of ecotoxicity tests in it [278].
- Ecodesign: reduce the environmental effects of products over their entire life cycle, an aim to be achieved by means of an appropriate design during product development.
- Quality by design: introduction of quality tests during the product development process leads to a more efficient fault identification and reduction.

In general, there are conflicting views about the existence and implementation of SbD in the production of NMs and nano-containing products. There are fields where SbD is more advanced due to specific requirements like nanomedicine. In this case, concepts or definitions like "immunity by design" [274], and "benign by design" [275] [269] are well defined and established, but this is not necessarily the case for nanotechnology in general. It has been claimed during the surveys that SbD for NMs exists and the tools and strategies have been developed and what is left is the implementation, testing, and validation in real life settings. These tools have been published in a peer reviewed special issue[2] of the NanoImpact journal, but the successful transfer from a scientific setting in real-world cases outside of research projects is a separate task by itself.

Specific approaches on the SbD of NMs include metal doping, surface coating and covalent functionalisation, and adjustment of surface oxidation state and aspect ratio of engineered [295] [9] [296]. The main techniques used are [9]:

- Coating (of which there were mentioned numerous examples above) and encapsulation
- Loading
- Grafting
- Doping

Similarly, specific principles for safer design of nanomedicines include [9]:

- Optimising the Size and Structure of Nanomaterials
- Regulating Nanomaterial-Related Perturbation of Cellular Redox Equilibrium
- Passivating Defect Sites of Nanomaterials
- Reducing Interaction between Nanomaterials and Biomolecules
- Preventing the Leakage of Toxic Components
- Controlling the Biopersistence of Nanomaterials
- Introducing Stimuli Responsiveness

Of key interest is the concept of safe-by-degradation, which implements the concept of optimised life-time of a NM to SbD including its safe clearance from the body and the environment [297]. This can be achieved, in principle, using several SbD strategies, with surface modification and functionalisation being key, as it can be structured to include "degradation initiation centres" in the form of specific defects [297]. Other approaches that include the concept of SbD are integrated approaches for testing and assessment of NMs [297], a toolbox of biophysical and functional *in vitro* assays for the suitability assessment of nanomaterials in the early stages of vaccine development [298], SIA (Safe Innovation Approach) [280] and AOP (Adverse Outcome Pathways) [281]. There has also been the suggestion for the establishment of a European Centre "to meet the needs of industry and other parties concerned with the safe and responsible innovation of nanotechnology, by establishing a one-stop shop for a wide variety of nanosafety related services" [299].

There are several limitations mentioned for the successful implementation of SbD in real life practice. First and foremost, the complexity of NMs regarding their properties and behaviour that

[2] https://www.sciencedirect.com/journal/nanoimpact/special-issue/10S1MKJ8583

can change in different environments makes it difficult to implement a unique model, which also makes it hard to implement traditional SbD approaches without modifications as described in section 4.2.4. As described in section 4.2.5 specific modifications include:

- Quick screening of large amounts of nanomaterials by HTS (high throughput screening) techniques, would be useful for more straightforward SAR (structure-activity relationships) [356].
- Need for assessment of the exact effective cellular dose of NMs compared with routine testing of soluble chemicals [306].
- Information on internal concentrations (also referred to as internalised dose) would add value and consequently, would be useful to inform SbD methods [304]. In general, this dose-response issue is one of the most challenging issues in the determination of the safety and toxicity of NMs.
- Modifications of existing biomarker screens could include the use of phagocytosis and endocytosis-related assays. [358]
- There is a need to modify the Organization for Economic Cooperation and Development (OECD) tests to enhance the concept that removal and uptake into the organism need to be considered as an important part of bioaccumulation and consequently of toxicity [359].
- There should be standardisation and validation of specific *in vitro* assays mimicking defined *in vivo* endpoints [360].
- There is a limited ability of the present *in vitro* assays to deal with secondary toxic mechanisms and organ specificity.
- Understanding in detail the mechanisms underlying inflammasome activation by various NMs may allow for refined "safe-by-design" approaches [361].
- There are needed modifications taking into consideration, challenges related to NM physicochemical properties and their interaction with surrounding and target environments, cellular uptake and novel endpoints, particularly epigenetics [326].

As also stated during the surveys, different criteria need to be established for their evaluation, while considering potential financial and political issues and the need for cultural change from stakeholders. This is especially the case for SMEs and start-ups who may have not the necessary infrastructure, processes, and funds to implement such strategies [300], or are unwilling to invest in SbD without knowing the profitability of a product in the R&D phase. Furthermore, substantial consensus is required among the different stakeholders and policy makers for the establishment and implementation of SbD strategies at a political level [301], [302]. It has been reported that some industries may believe that they are applying or implementing SbD strategies, while it may not be the case, although no specific results have been offered.

This is especially true considering that consensus exists on the need for re-evaluation of NMs if such processes are to be implemented even in regulatory requirements, including updated guidelines, even if very sector specific as it was suggested. In any case, it has been expressed that SbD is a concept that should be part of regulatory requirements but should not substitute thorough regulatory evaluation. It was suggested by one participant that as soon as there exists a set of concise, robust, and proven SbD criteria for a given NM, a respective framework regarding the use of these NMs should be developed with guidance on how to regulatory conform with these criteria. On the other hand, there were two participants that stated that SbD should not be part of the regulatory framework, with the rest offering no opinion or referring to incentives for industry to implement such practices.

One of the key challenges regarding the SbD of NMs is the lack of high-quality scientific data that can assist with developing alternative testing strategies and lead to the development of robust and validated computational workflows that can be accepted under a regulatory framework [304] [310] [307]. Such approaches can help with the design and development of new NMs since they are able to predict either the properties or the behaviour of a NM under different conditions. Other approaches include high-throughput screening and omics. All these

approaches need to focus on nanospecific descriptors, of both physicochemical parameters but importantly also biological data. Here it is specifically important to identify differences to 'classical' materials specifically with the focus on exposure - and here specifically occupational exposure which remains the current hotspot - as the toxicity is driven by their size, form and shape resulting in potentially exerted adverse effects. This means that similarly to (bio)degradation, current methodologies may need to be adapted specifically in terms of qualitative and quantitative detection limits to be able to be used in such studies. Another challenge for the development of complete SbD strategies is the study and understanding of secondary genotoxicity, which is based on immune cells such as macrophages and neutrophils recruited to clear the tissue from foreign NMs leading to ROS and is currently challenging to predict, due to deficiencies in standard monoculture systems [309].

6. References

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7. Annex

7.1 (Bio)degradation of NMs and organic coatings questionnaire

7.1.1 Introduction

Dear Colleague,
We are conducting a survey on the (bio)degradation of nanomaterials and organic coatings on behalf of the EU Observatory for Nanomaterials (EUON) and the European Chemicals Agency (ECHA). The aim is to improve the transparency of information on the safety and markets of nanomaterials.
The key aim of this study is to address the degradation of nanomaterials (including biodegradation where relevant, for either organic nanomaterials or organic coatings on nanomaterials). In addition, the study aims at examining what tools are available for the assessment of (bio)degradation, and how these can be used in different regulatory processes.
Therefore, we are sharing this questionnaire, which should not take more than 15 minutes to complete, to help us gather the necessary information. We would appreciate your participation in our study. Following participation, if you wish, you can gain early access to the report that we will compile with EUON/ECHA. In any case, any information shared with us will be treated, if desired, as confidential and will not be published.
Questionnaire link: https://novamechanics.com/biodegradation-of-nanomaterials/
If you don't wish to fill in the questionnaire and would prefer a live interview, we would be happy to arrange this with you. Please mail us at XXX@novamechanics.com
Thank you very much in advance for your time and participation.
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7.1.2 Section 1 Personal information

- Name:
- Organisation
- Position:
- Email:
- Phone number:

7.1.3 Section 2 (Bio)degradation definition

1. Are you currently studying or have studied the (bio)degradation of nanomaterials? Yes/No
    a. If yes, what is the type of nanomaterials that you worked with?
2. How would you define nanomaterials degradation?
    a. Does this definition fit the degradation of organic coatings?
    b. If no, how would you define it for organic coatings?
3. How would you define nanomaterials biodegradation?
    a. Does this definition fit the biodegradation of organic coatings?
    b. If no, how would you define it for organic coatings?
4. Do you believe that a common definition for nanomaterials (bio)degradation is possible?
    a. If yes, what would be a possible definition?
    b. If no, which nanomaterials could be possibly grouped together under a common definition?
5. Should any provided information be treated as confidential? Yes/No
    a. If yes, please tell us which.

7.1.4 Section 3 (Bio)degradation strategies and techniques

6. Are you aware of specific approaches/strategies used for the study of (bio)degradation for nanomaterials?
    a. If yes, which are these techniques?
7. Are you aware of specific approaches/strategies used for the study of (bio)degradation for organic coatings?
    a. If yes, which are these techniques?
8. Are you aware of specific techniques used to monitor the (bio)degradation for nanomaterials?
    a. If yes, which are these techniques?
9. Are you aware of specific techniques used to monitor the (bio)degradation for organic coatings?
    a. If yes, which are these techniques?
10. In your opinion, can strategies and/or techniques used for the study/monitoring of traditional/bulk chemicals used for the study of (bio)degradation of nanomaterials? (yes, no, with modifications)
    a. What modifications would be needed to be able to use such techniques for the study of nanomaterials (bio)degradation?
11. In your opinion, can strategies and/or techniques used for the study/monitoring of traditional/bulk chemicals used for the study of (bio)degradation of organic coatings? (yes, no, with modifications)
    a. What modifications would be needed to be able to use such techniques for the study of nanomaterials (bio)degradation?
12. Should any provided information be treated as confidential? Yes/No
    a. If yes, please tell us which.

7.1.5 Section 4 (Bio)degradation and regulation

13. Are you aware of standardised techniques and/or approaches existing for the study of (bio)degradation of nanomaterials and/or organic coatings?

    a. If yes, which are these techniques?
14. Are you aware of techniques and/or approaches existing for the study of (bio)degradation of nanomaterials and/or organic coatings that are accepted in a regulatory context?
    a. If yes, which are these techniques?
15. Which existing standardised techniques and/or approaches can be used for the study of (bio)degradation of nanomaterials and/or organic coatings?
    a. What modifications, if any, would be required?
16. Which existing regulatory guidelines can be used for the study of (bio)degradation of nanomaterials and/or organic coatings?
    a. What modifications, if any, would be required?
17. Are you aware of any ongoing actions for the development of standardised techniques and/or strategies for the study of the (bio)degradation of nanomaterials and/or organic coatings?
    a. If yes, can you please tell us more?
18. Should any provided information be treated as confidential? Yes/No
    a. If yes, please tell us which.

7.1.6 Section 5 (Bio)degradation gaps and future steps

19. Which do you think are the current gaps for the study of nanomaterials (bio)degradation?
20. Which do you think are the current gaps for the study of (bio)degradation of organic coatings?
21. Where do you think research should focus?
22. Do you think that public funding is required for the study of (bio)degradation and/or organic coatings?
23. Should any provided information be treated as confidential?
    a. If yes, please tell us which.
24. Would you be interested to participate in a focus group regarding the (bio)degradation of nanomaterials and/or organic coatings? Yes/No
    a. If yes, can we contact you to discuss further?
25. Would you be interested to give us a personal perspective regarding the (bio)degradation of nanomaterials and/or organic coatings? Yes/No
26. If yes, can we contact you to discuss further?

7.2 SbD of NMs questionnaire

7.2.1 Introduction

Dear Colleague,
We are conducting a survey on the (bio)degradation of nanomaterials and organic coatings on behalf of the EU Observatory for Nanomaterials (EUON) and the European Chemicals Agency (ECHA). The aim is to improve the transparency of information on the safety and markets of nanomaterials.
Another key aim of the study is to examine the current state of the art for safe by design (SbD) of nanomaterials, as it relates to the degradation and persistence of nanomaterials. We aim to examine how SbD of nanomaterials considers the (bio)degradation of nanomaterials and provide recommendations to adapt existing SbD principles to consider the knowledge collected from literature on (bio)degradation of nanomaterials to reduce their persistence in the environment.
Therefore, we are sharing this questionnaire, which should not take more than 20 minutes to complete, to help us gather the necessary information. We would appreciate your participation in our study. Following participation, if you wish, you can gain early access to the report that we will compile with EUON/ECHA. In any case, any information shared with us will be treated, if desired, as confidential and will not be published.
SbD survey link: https://novamechanics.com/safe-by-design-of-nanomaterials/
If you don't wish to fill in the questionnaire and would prefer a live interview, we would be happy

to arrange this with you. Please mail us at XXX@novamechanics.com
Thank you very much in advance for your time and participation.
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7.2.2 Section 1 Personal information

- Name:
- Organisation
- Position:
- Email:
- Phone number:

7.2.3 Section 2 SbD definition

27. Are you currently studying or have studied the SbD of nanomaterials? Yes/No
    a. If yes, what is the type of nanomaterials that you worked with?
28. How would you define SbD for nanomaterials?
29. Do you believe that a common definition for nanomaterials SbD is possible?
    a. If yes, what would be a possible definition?
    b. If no, which nanomaterials could be possibly grouped together under a common definition?
30. Do you think that the SbD of nanomaterials exists?
    a. Examples?
31. Do you think that SbD of nanomaterials is being implemented in nanomaterials production?
    a. Examples?
32. Should any provided information be treated as confidential? Yes/No
    a. If yes, please tell us which.

7.2.4 Section 3 SbD strategies and techniques

33. Are you aware of specific approaches/strategies used for the SbD for nanomaterials?
    a. If yes, which are these techniques?
    b. What are their boundaries?
34. Are you aware of specific techniques used in SbD for nanomaterials?
    a. If yes, which are these techniques?
    b. What are their boundaries?
35. In your opinion, can strategies and/or techniques used for the study/monitoring of traditional/bulk chemicals used for the SbD of nanomaterials? (yes, no, with modifications)
    a. What modifications would be needed to be able to use such techniques for the SbD of nanomaterials?
36. Are you aware of specific ongoing actions studying the SbD of nanomaterials?
    a. If yes, which are these techniques?
37. Are you aware of specific SbD strategies of nanomaterials used in industry?
    a. If yes, which are these techniques?
38. Should any provided information be treated as confidential? Yes/No
    a. If yes, please tell us which.

7.2.5 Section 4 SbD and regulation

39. Are you aware of standardised techniques and/or approaches existing for the SbD of nanomaterials?
40. Are you aware of techniques and/or approaches existing for the SbD of nanomaterials that are accepted in a regulatory context?
41. Which existing standardised techniques and/or approaches can be used for the SbD of nanomaterials?
    a. What modifications, if any, would be required?
42. Which existing regulatory guidelines can be used for the SbD of nanomaterials?
    a. What modifications, if any, would be required?

43. Will the use of modified strategies for the SbD of NMs lead to the need for their re-evaluation under a regulatory context? (Yes, No, Partly)
44. Are you aware of any ongoing actions for the development of standardised techniques and/or strategies for the SbD of nanomaterials?
    a. If yes, can you please tell us more?
45. Should any provided information be treated as confidential? Yes/No
    a. If yes, please tell us which.

7.2.6 Section 5 SbD gaps and future steps

46. Which do you think are the current gaps for the SbD of nanomaterials?
47. Which are the emerging strategies for the SbD of nanomaterials?
48. Where do you think research should focus?
49. Do you think that public funding is required for the SbD of nanomaterials?
50. Should SbD be part of regulatory requirements?
51. Can public communication of SbD strategies help with the societal acceptance of NMs-containing products?
52. Should any provided information be treated as confidential?
    a. If yes, please tell us which.
53. Would you be interested to participate in a focus group regarding the (bio)degradation of nanomaterials and/or organic coatings? Yes/No
    a. If yes, can we contact you to discuss further?
54. Would you be interested to give us a personal perspective regarding the (bio)degradation of nanomaterials and/or organic coatings? Yes/No

If yes, can we contact you to discuss further?