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ACEnano

Analytical and Characterisation Excellence

Analytical and Characterisation Excellence in nanomaterial risk assessment: A tiered approach

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ACEnano's NANO-METHOD DECISION TOOL – A TOOL WITH UNIQUE FEATURES TO SUPPORT SELECTION OF SUITABLE TECHNIQUES FOR NANOMATERIALS' CHARACTERIZATION.

SIMPLE GUIDE FOR SMEs

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FOREWORD

ACEnano, a H2020 EU funded project named "*Analytical and Characterization Excellence in Nanomaterial risk assessment: A tiered approach*" has been actively working since 2017 on introducing confidence, adaptability and clarity into nanomaterial risk assessment by bringing innovative solutions in nanomaterial characterisation, along with the creation of support structures to facilitate analytical decisions. These support structures include the development of a "Nano Method-Decision Tool" which facilitates the analytical characterisation of nanomaterials. The "Simple Guide for SMEs" is designed to guide and support SMEs, when using the tool.

The guide includes a brief introduction with relevant topics that highlights the importance of why a deep knowledge about nanomaterials is so critical and what needs to be taken into account during the process of translation of innovative products into the market, in order to ensure confidence in their functionality and safety. The guide contains information on how to access and use the tool, as well as a brief description of the techniques that are included in the tool, linked to explanatory videos. Together with the guide, the tool can help users identify the right information about the most appropriate characterisation methods required.

Finally, the guide provides information on experts and facilities where relevant nanomaterial characterization techniques are available, to help users if further support is required.

WHAT IS A NANOMATERIAL?

There are several international definitions of nanomaterials (NMs) and nanoparticles (NPs), that have been proposed by governments, industry, and standardisation organisations, and which currently remain in use despite this ambiguity.

The definition that ACEnano applies when referring to a NM is based on the one that the European Commission[1] states for NM as:

> ***"A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm. In specific cases and where warranted, by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %. By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials."***

NMs have brought and continue to bring promising advances to science and technology. Regardless of the definition that one applies, it is more important that there is an obligation to ensure that a NM is evaluated appropriately when determining whether it poses a risk to human health or the environment. This evaluation should be based not only on the intrinsic hazard potential of the NM, but also on the consideration of its exposure potential (e.g. during manufacturing, use, and its disposal)[2].

The number of different types of NMs which are currently exploited and their broad application range in everyday products is increasing very fast. While industry, regulatory bodies, society and governments are aware of this, also the concern about potential risk of these NMs is increasing. Therefore, in recent years immense efforts have been dedicated in the development of new analytical techniques and protocols as well as combinations of existing ones. Moreover, support structures to facilitate analytical decisions have also been developed. All these efforts combine to provide an improved detection, quantification and characterisation framework for the safer use of NMs found in commercial products and having the

[1] European Commission. Commission Recommendation of 18 October 2011 on the definition of nanomaterial, 2011/696/EU. L275, 38–40 (Official Journal of the EU, 2011).

[2] http://dx.doi.org/10.1016/j.yrtph.2015.06.001

potential to reach humans and the environment. ACEnano has contributed to this field, by inventing, assembling, assessing,

adapting and improving a list of methods and techniques (See **Table 1** and **Annex I**) that help to provide knowledge about and confidence in the physico-chemical characterisation of the full range of NM variability (i.e., including commercially available NMs, legacy materials and novel not yet commercial 2nd generation NMs).

NANO-RELEVANCE AT A GLANCE:

Why is the targeted characterisation of a NM important?

NMs are a growing class of materials for which interest is increasing rapidly due to their unique size-related properties relevant to a range of novel applications. Great expectations surround the potential of engineered NMs to confer a competitive advantage in the development of innovative products. The range of products potentially containing NMs covers already all types of daily-use products such as textiles, sport devices, nano-pharmaceutical products, nano-medical devices, cosmetics, biocides or even toys[3]. Furthermore, NMs are also broadly used as additives, in e.g., colour inks or paints, plastics or steel compounds[3].

However there are uncertainties remaining in relation to their behaviour, for example:

i) *How do NMs* ***interact with biological and environmental systems****?* ***ii)*** *What are their* ***specific risks to humans and the environment****?* ***iii)*** ***How safe and sustainable are*** *products containing NMs?* ***iv)*** *Are there* ***legal issues*** *to be taken into account?*

To be able to answer such questions, it is mandatory to have a deep knowledge about the NMs used in the development of a given product or application. Such knowledge begins with an appropriate characterisation of the NMs, becoming the starting point for the description of a certain NM, potential risks related to its use and the determination of appropriate risk management measures. A throughout characterisation of NMs is essential for establishing effective product quality control measures, as well as for regulatory purposes that any new product needs to undergo and comply with, on its way to market.

Unlike bulk materials, the complexity in reliable characterisation grows when dealing with NMs. Most of bulk materials can be described sufficiently when their concentration and elemental composition are known. On the contrary NMs display size-related variation in their properties, whether they are found in suspensions, powders, thin films, or any other form and their behaviour may change dynamically as a function of their surroundings or time[4].

A deep and thorough characterisation of a NM is a challenging task and warrants the utilisation of state-of-the-art instrumentation and methods. Several techniques can be used to characterise the size, the crystal structure, elemental composition and a variety of other physical and chemical properties, depending on contextual requirements. Moreover, a specific physico-chemical property can be evaluated by more than one technique. The different strengths and limitations of each technique complicate the choice of the most suitable method, while often a combinatorial characterization approach is the needed option[5]. This may raise again some questions such as:

i) ***What*** *are the most appropriate* ***methods and/or techniques*** *to characterise a specific NM?* ***ii)*** ***What kind of equipment and know how is required*** *to perform these analyses? And as a consequence* ***iii)*** *Can*

[3] https://product.statnano.com
[4] http://dx.doi.org/10.1021/nn901112p
[5] https://doi.org/10.1039/C8NR02278J

*these analyses be done in house**?*** *and if not* ***iv)*** *How and where can* ***feedback or support from an expert*** *be found?*

The future of nanotechnology is based on approaches to develop new, useful NMs and to test them in relevant, potentially complex systems. This process should end up with new and innovative products reaching the market.

There is an opportunity and a need to strike a new balance that drives high quality research, simplifies commercial exploitation and enables reasoned regulatory approaches[6].

ACEnano has developed several interconnected decision tools to help users to have access to and knowledge about the relevant tools, methods, instrumentation and suppliers that support them in the development of safe new NM and/or NM-containing products with desired features.

Evaluating the risks and benefits when including or using NMs in a new product:

Society is increasingly aware of the wide use and applications of NMs, but there is very limited understanding on how the use of NMs brings benefit to different technologies or products, and ultimately to the users and consumers. Most people link the use of NMs to electronics, some surface treatments, cosmetics, and textiles. As the public becomes increasingly aware that human health also reflects, to a certain extent, lifestyle choices, and as consumers show a growing interest in environmental issues, also becoming more cautious when buying goods. They are more interested in knowing the origins and content of the products they buy as they can impact their safety, their health and the environment[7].

Societies as a whole perceive health risks as an important issue for the life of their citizens. The public perception of risks and benefits associated with NMs is highly variable and depends in most cases on the applications or types of products in which these NMs are used. This risk-benefit perception towards NMs seems to be associated with the people's expected level of exposure to the NM arising from different sources. Food contact materials or cosmetic products such as sunscreens, for example, raise higher concerns in most studies than products where lower/no or indirect exposure to NMs is expected, e.g., computers. The level of consumers' concern increases with the anticipated increasing probability to direct exposure to NMs, where the concerns are about potential internalisation of the NMs (with dermal exposure being the most likely route)[6]. Their concerns are mainly associated with what they perceive as yet to be discovered impacts and properties of NMs and limited means to avoid exposure. However, negative impacts can be avoided or prevented by proper development, use and/or treatment of NMs. Again, a deep and throughout characterization of a NM is essential here as it will provide meaningful answers and may thus mitigate these concerns by giving the information on how a NM interacts with its surroundings (e.g., in contact with biological fluids, tissues, etc.), how stable it is, how it may potentially degrade, etc.

The legal-framework around NMs - a brief introduction:

In the EU, NMs are covered by the same rigorous regulatory framework that ensures the safe use of all chemicals and mixtures, i.e., the REACH[8] (Registration, Evaluation, Authorisation and Restriction of Chemicals) and CLP[9] (Classification, Labelling and Packaging) regulations. REACH places the burden of proof on companies. To comply with the regulation, companies must identify and manage the risks linked to the substances (also of NMs) they manufacture and market in the EU. They have to demonstrate to the ECHA[10] (European Chemical Agency) how the substance can be safely used, and they must communicate the risk management measures to the users.

[6] ACSNano: d.o.i:009. 10.1021/nn901112p CCC

[7] Understanding public perception of nanomaterials and their safety in the EU. Final Report November 2020. DOI 10.2823/82474

[8] https://echa.europa.eu/regulations/reach/understanding-reach

[9] https://echa.europa.eu/regulations/clp/legislation

[10] https://echa.europa.eu/

All in all, this means that hazardous properties of NMs or substances have to be assessed and their safe use needs to be ensured.

Moreover, there are specific provisions for NMs in sector-specific legislation such as food, biocides and cosmetics, plant protection and pharmaceuticals' legislation.

➔ In the case of the ***food sector***, in the EU, the EFSA[11] (European Food Safety Authority) is responsible for the risk assessment of NMs used in food and feed, as well as in food contact materials. With the increased use of NMs in the food and feed chain, it is very important to know the properties and characteristics of NMs and determine whether they raise any potential health or environmental concerns. Therefore, in the EU several regulations have already been installed in order to specifically cover the use of NMs in this sector, introducing novel concepts and their specific regulation like the Novel Food Regulation[12], the Food Additives Regulation[13], the Food Information to Consumers (FIC) Regulation[14] (under this regulation, all ingredients that are engineered NMs need to be clearly indicated in the list of ingredients), the Plastic Food Contact Materials Regulation[15] and the Active and Intelligent Food Contact Material Regulation[16].

➔ In the ***cosmetics sector***, the EU Cosmetics Regulation[17] is the one in place to safeguard consumer health, but also underpinning European innovation and strengthening the competitiveness of the cosmetics sector at the global level. The inclusion of a NM in a cosmetic product must be explicitly stated when the cosmetic product is notified to the CPNP[18] (Cosmetics Products Notification Portal) before being placed on the EU market, and it must contain the following information: i) identification of the NM (chemical name and other descriptors), ii) specification of the NM, including particle size and physical and chemical properties, iii) estimation of the quantity of the NM contained in the cosmetic product per year, iv) a toxicological profile of the NM, v) the safety data of the NM and vi) all information related to foreseeable exposure conditions.

➔ When talking about ***biocidal products***, a dedicated risk assessment is needed when a NM is used as active and non-active substance and the Biocidal Product Regulation[19] has specific provisions for NMs covering this issue.

➔ In the ***MedTech sector***, Medical Devices (MDs) and In Vitro Diagnostic Medical Devices (IVDs) need to be certified with a CE mark before entering the market. It is a Notified Body, the responsible accrediting organ to issue a conformity certificate, declaring whether the specific product meets the applicable essential technical requirements, publicised in the MDs and IVDs regulation[20-21]. With this declaration of conformity, the manufacturer can label the product with the CE Mark, and therefore can enter into the market and its distribution and sale is allowed in the EU. In May 2021 the new EU regulation on Medical Devices[20] (MDR) has entered into force and the EU regulation on In Vitro diagnostic Medical Devices[21] (IVDR) will follow in May 2022. Both regulations give special attention to NMs and define the specific requirements that MDs or IVDs need to be fulfilled when NMs are used in their design and manufacture. They specify the requirement of reducing, as far as possible, any risks linked to the size and the properties of NPs and NMs which are or can be released into the user's body. These new regulations will create a robust, transparent and sustainable regulatory

[11] https://www.efsa.europa.eu/en
[12] Novel Foods Regulation (EC) No 2015/2283
[13] Food Additives Regulation (EC) No 1333/2008
[14] Food Information to Consumers Regulation – (EC) No 1169/2011
[15] Plastic Food Contact Materials Regulation (EC) No 10/2011
[16] Active and Intelligent Materials and Articles Regulation (EC) No 450/2009
[17] Cosmetics Regulation (EC) No 1223/2009
[18] Cosmetic Products Notification Portal (CPNP)
[19] Biocidal Product Regulation (EU)
[20] Regulation on Medical Devices (EU) 2017/745
[21] Regulation on in vitro diagnostic medical devices (EU) 2017/746

framework, accepted internationally, that improves clinical safety and will create a fair market access for manufacturers. Unlike directives, regulations do not need to be translated into national laws, and therefore the MDR and the IVDR will mitigate discrepancies in interpretation across the EU market.

➔ Finally, the EU legislation[22,23,24] on ***worker protection*** applies to chemicals and therefore also to NMs, although it does not refer explicitly to those. By law, employers are required to assess and manage the risks of chemicals, including NMs, at work and actions should be taken to remove or reduce the risks as far as possible.

THE NANO METHOD-DECISION TOOL – A SHORT OVERVIEW

The development of the Nano Method-Decision Tool is based on a previous existing tool named the NanoDefiner e-tool[25]. The user can access the Nano Method-Decision Tool via http://www.acenano-project.eu/. The tool is designed to guide users through a catalogue of dedicated questions with the aim to eventually provide a list of appropriate analytical techniques for the characterisation of their specific NM.

Categories:

The user of the Nano Method-Decision Tool has three different types of categories available that can be of interest, depending on the purpose of the characterisation required for the specific NM. For these categories a set of parameters has to be defined. The three categories and their specific parameters considered by the tool are:

i) **Regulation:** there are different descriptors of NM under evaluation that may need to be determined. The descriptors are: i) size & size distribution, ii) shape (aspect ratio), iii) surface functionalisation and iv) surface area.

ii) **Risk Assessment:** as for the regulation category there are different descriptors of a given NM that may need to be evaluated in order to have the relevant information about the risk of the NM under consideration. These parameters are: i) solubility, ii) shape (aspect ratio), iii) surface reactivity, iv) surface area, v) size & size distribution, vi) agglomeration, vii) surface coating and viii) surface charge.

iii) **Labelling:** depending on the matrix in which the NM is embedded, the user can choose among: i) cosmetics, ii) biocides and iii) food.

By selecting any of the previous categories and specific parameters or descriptors that the user needs to determine, the tool, automatically, shows a window containing a list of questions, that once answered, essentially provide a description of the material properties. This description allows the tool to recommend only those techniques (or only one single technique) from an initial list of "potential techniques" that are suitable to determine the desired descriptors or parameters of the NM.

Thereby, the tool answers as a traffic-light like system to indicate the suitability of the different technique/s. The user can directly see what happens when choosing different options to answer given questions. This interactive modus of the tool may provide guidance for the user, when more than one option is possible. Once the tool discards a technique for not being suitable, it automatically highlights it in red colour. If the tool considers a technique to be suitable, but not enough information is available to provide a reliable assessment, it is indicated in orange. Once a technique has been chosen as the best one (more than one technique can be chosen) they are highlighted in green.

[22] Framework Directive 89/391/EEC
[23] Chemical Agent Directive 98/24/EC
[24] Carcinogen and Mutagen Directive 2004/37/EC
[25] NanoDefine Project. Catalogue of technical methods performance criteria. Deliverable number 7.1.

Once the best techniques have been displayed by the tool, the user can also take advantage of another feature of the tool that is the direct interconnection with the ACEnano Knowledge Warehouse[26]. To do so, the user only needs to click on top of the chosen technique on a button named "Info". By clicking on it, the Nano Method-Decision Tool re-directs the user to the specific site of the technique description on the ACEnano Knowledge Warehouse platform (*for more information see ACEnano's Knowledge Warehouse section in this guide*). Once there, the user can find relevant and detailed information related to the technique the Nano Method-Decision tool was indicating, in an intuitive and easy way. The information that can be consulted in this platform is:

i) *A technical description of the technique,* ***ii)*** *the endpoints of the technique,* ***iii)*** *the measurement protocols available for the technique,* ***iv)*** *sample preparation protocols available for this technique,* ***iv)*** *datasets that have been uploaded by users and if they are open access or not, as well as* ***v)*** *respective providers.*

For a more detailed description and explanation about the exact operation and features of the Nano Method-Decision Tool the user can visit the following link: https://youtu.be/gwOsAZ-zDmI?t=1666. This video summarizes in a very detailed way the main features of the tool.

Techniques under consideration in the Nano Method-Decision Tool:

The Nano Method-Decision tool takes into consideration a broad variety of analytical techniques with their individual advantages and challenges. Within this guide an overview of these respective techniques is provided. In this context, **Table 1**[27] contains a brief description of the techniques that the tool considers, the type of information that the user gets from each technique and a link to explanatory videos for each technique.

"The Nano Method-Decision tool - a tool with unique features connected to the ACEnano Knowledge Warehouse"

ACEnano KNOWLEDGE WAREHOUSE

As mentioned in the section above, the Nano Method-Decision Tool is interlinked with the **ACEnano Knowledge Warehouse.** This is a publicly accessible data infrastructure, developed within the ACEnano project that can be used by any other organisation or project, but specifically by users or companies looking for state-of-the-art information related to NMs research and characterisation. The ACEnano Knowledge Warehouse stores and shares data and protocols created by specialised laboratories and companies that are part of the ACEnano project, most of them created and validated during the project lifetime.

By consulting this knowledge infrastructure, method developers (e.g., instruments providers, laboratories working on the development of new NM characterisation methods) can store detailed protocols and corresponding data to document the optimisation and validation of their techniques. This in turn helps and guides methods' applicants (e.g., industry or research laboratories) to have access to existing procedures, workflows or datasets and evaluate them, as well as learn to apply them by themselves. The ACEnano Knowledge Warehouse is designed to provide access to harmonised and standardised methods and data, implementing FAIR (findable, accessible, interoperable and reusable) data principles, that secure the reproducibility and documentation process towards the goal of generating reference resources for NMs risk assessment.

[26] The ACEnano Knowledge Warehouse is a publicly accessible data infrastructure that contains state-of-the-art information related to NMs research and characterisation.

[27] *The 20 techniques of the Nano Method-Decision Tool are only a part of all the techniques that can be found in ACEnano's Knowledge Warehouse. They are the most commonly used and for which SOPs or at least well-defined protocols are already available.*

The main features offered by the Knowledge Warehouse include:

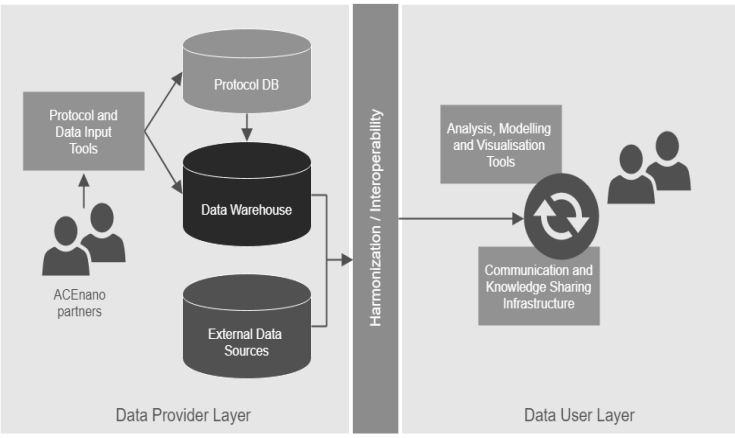

***Figure 1**: Schematic representation of the ACEnano Knowledge Warehouse structure.*

- A simple and data protected log-in system;
- A space for addition, storage and sharing of protocols and procedures;
- Access to complete physicochemical characterisation workflows (from sample preparation to measurement and data treatment);
- Upload and download raw, processed and summary datasets;
- Harmonisation of methodologies within organisations or projects;
- Support to intra- and interlaboratory comparison of protocols and results towards achieving reproducibility and validation goals;
- Automatic use of data for analysis and computational modelling via the application programming interface (API);

How can a user access the Knowledge Warehouse[28] and how does it work?

The Knowledge Warehouse can be accessed through the http://www.acenano-project.eu/ web address. The user can decide to generate a specific account on the platform or use one of his social media accounts. The Knowledge Warehouse is organised in a technique-centered way and contains:

1. An **entry page** to the platform: it presents the ***protocols and data available, grouped by technique***,

2. A ***catalogue of techniques and endpoints*** section, giving a short description of the techniques, their development status and availability and links to more detailed descriptions and related standard operating procedures (SOPs),

3. Available ***search features*** **for protocols and data sets** based on techniques and endpoints.

Thus, in brief:

The features of the **ACEnano Knowledge Warehouse allow** its users to:

i) browse, view, create, store and share protocols *on physico-chemical characterisation of NMs;* ***ii) browse, view, upload, store and share*** *NMs physicochemical characterisation* ***data****,* ***iii) track*** *the experimental* ***results to the protocols used*** *to generate the respective datasets, and what is of upmost importance,* ***iv)*** *it permits to give the user a* ***fast and easy way to know*** *the* ***technical details and detailed information of the techniques*** *that are more suitable for the characterization of a given NM.*

Apart from being linked to the Nano Method-Decision Tool, the ACEnano Knowledge Warehouse is composed of four types of sections that are interconnected. These sections are:

➔ the ***"Techniques' Catalogue"*** section: it contains 57 different techniques and related endpoints covered by the ACEnano project. By clicking on each of them, more detailed information like benefits,

[28] *To learn more about the main functionalities of the Knowledge Warehouse click on ACEnano Knowledge Infrastructure Manual (available at https://acenano.douglasconnect.com/about/).*

targeted market sector, targeted activity and explanatory videos can be found. (A list of all these techniques and link to the Knowledge Warehouse can be found in **Annex I** of this guide).

- ➔ the ***"Protocols"*** section: it compiles all protocols (methods) used or developed within the ACEnano project for each of the techniques mentioned in the point above. It contains protocols that can be used for sample preparation, measurements or data treatment.
- ➔ the ***"Data"*** section: the platform offers long-term storage of data produced by the nanosafety community and supports the required data harmonisation and FAIR principles implementation in order to generate a reference resource for NM risk assessment.
- ➔ the ***"Events & Publications"*** section: it facilitates further dissemination of the activities and outcomes of the Knowledge Warehouse to the scientific community as well as to the general public or any user of the platform.

ON THE SEARCH FOR THE RIGHT EXPERT & PARTNER FOR A SPECIFIC NEED.

Once the user has gained the necessary knowledge about which is the best technique to characterise a given NM, a user may consider different options in order to characterise the NM under evaluation. The user may decide:

a) *To perform the needed physico-chemical characterisation assays by itself*. In order to support the users to do so, as explained in the section above, the ACEnano Knowledge Warehouse offers a wide variety and different type of protocols and methods that the user can follow and/or consult. All relevant information to do so can be found at the ACEnano Knowledge Infrastructure Manual[26] that can be consulted by entering the platform through the ACEnano project website[29].

b) *To outsource the performance of the required physico-chemical characterisation.* A user may not have the knowledge and/or the instrumentation/facilities needed to perform the analyses needed to characterize a NM. This leads the user to search for experts that could provide him both, specific characterisation services and the right facilities and instrumentation to do so.

In order to tackle this last point, i.e., search for experts on NMs characterisation and facilities where to perform these types of analyses, this guide also envisages giving a brief overview about some entities the user may contact and ask for specific characterisation services, if needed.

Among all players in Europe that provide this kind of services (i.e., private companies, private and public research institutes, European funded R&D facilities or infrastructures, etc.), this guideline shows only some examples. Among the listed facilities and experts, partners of the ACEnano project that have been involved in the development of some of the protocols, methods, standardized operating procedures (SOPs), or data stored on the Knowledge Warehouse can be found (**See Annex IIa**). Furthermore, the guide also lists some other European experts and facilities that may also provide support to users (**See Annex IIb**).

[29] http://www.acenano-project.eu/

Table 1: List of analytical techniques that are included in the Nano Method-DecisionTool.

Technique	How it works	Information it provides
Transmission Electron Microscopy (TEM)	TEM is a high spatial resolution imaging and characterization tool. It is a microscopy technique in which a beam of electrons is transmitted through a sample to form an image. The sample is most often an ultrathin section less than 100 nm thick or a suspension on a grid. It requires theoretical insight and practical skills of a user but nevertheless TEM is a standard tool for measurements on the nanoscale and standard procedures exist.	- particle size and size distribution based on 2D images and image analysis, - shape, - homogeneity, - elemental composition (semi-quantitative), - chemical and crystallographic information.
	Explanatory Video: https://www.youtube.com/watch?v=fQJYuTpK8Fs	
Scanning Electron Microscopy (SEM)	This type of electron microscope produces images of a sample by scanning the surface of the sample with a fine probe of electrons. The electrons interact with atoms in the sample and generates signals that contain information about the surface topography and composition of the sample.	- the elemental composition of the sample (semi-quantitative), - the NP surface morphology - the NP size and size distribution, based on 2D images.
	Explanatory Videos: https://www.jove.com/v/5656/scanning-electron-microscopy-sem/, https://www.youtube.com/watch?v=uQ1gCIkCbIQ	
Atomic Force Microscopy (AFM)	An AFM generates images by scanning a small cantilever over the surface of a sample. A sharp tip (probe) on the end of the cantilever contacts the surface bending the cantilever and changing the amount of laser light reflected into the photodiode. The AFM has three major abilities: force measurement, topographic imaging, and manipulation. Moreover, this imaging tool has lateral and vertical spatial resolution in the low-nm range.	- the NP size distribution patterns and - the dimensions of individual NPs in the best case.
	Explanatory Videos: https://www.youtube.com/watch?v=8gCf1sEnoUU	
X-Ray-Diffraction (XRD) Powder-X-Ray-Diffraction (PXDR)	XRD is a widely used technique for analysis of the crystalline structure of materials. Its fundamental equation is based on the Bragg's law equation, which links lattice spacing with the observed angles of constructive interference when X-rays scatter from a crystalline material. XRD provides a powerful and relatively simple way of determining the average particle size of a NM, but not the size or distribution. The technique is more reliable for particles towards the lower end of the 1 nm to 100 nm size range.	- crystallinity: Percentage of crystalline and amorphous content – application in semi-crystalline polymers - phase Identification: Determination of the phase(s) contributing to the diffraction pattern – makes use of powder diffraction database. - qualitative determination: phase mixtures, polymorphs

		- lattice parameter: Determine the lattice parameter of phase –able to monitor changes with processing or incorporation of dopants in lattice - crystallite size / strain: Leads to broadening of diffraction peaks - crystallite size sometimes equivalent to nanoparticle size
	Explanatory Videos: https://www.jove.com/v/10462/single-crystal-and-powder-x-ray-diffraction, https://www.jove.com/v/10446/x-ray-diffraction, https://www.youtube.com/watch?v=QHMzFUo0NL8	
Small Angle X-Ray Scattering (SAXS)	It is a method for determining the average sizes and shapes of nanoobjects in the typical range from 1 to 100 nm. A broad range of materials can be measured including polymers, colloids, nanocomposites, metals, minerals, biological materials, food and pharmaceuticals. Thereby, nanoparticles in solid and liquid matrices can be measured, but also porous materials. No elaborate sample preparation is needed. Additionally, no standards are required for the determination of the object size and structure.	- average size or shape - particle number density - mean molecular weight (with a calibrated intensity scale).
	Explanatory Videos: https://www.jove.com/v/58538/structural-studies-macromolecules-solution-using-small-angle-x-ray, https://www.youtube.com/watch?v=Vfn--l3xkWw	
Solvent Relaxation NMR	It is commonly used for the identification of unknown compounds, while the use of proton relaxation rates allows the surface area to be obtained for any particle of any size or shape. The NMR relaxometry is based on the observation that bound liquid in contact with a particle surface has a relaxation rate shorter by orders of magnitude from that of free liquid.	- in situ measured specific surface area
	Explanatory Videos: https://www.jove.com/v/5680/nuclear-magnetic-resonance-nmr-spectroscopy	
Brunauer-Emmett-Teller (BET)	This theory explains the physical adsorption of gas molecules on a solid surface and serves as the basis for an important analysis technique for the measurement of the specific surface area of materials. Nowadays, it consists of the adsorption of a monolayer of gas (usually nitrogen, but krypton, argon and carbon dioxide are also used) on the surface of objects constituting the powdered sample.	- specific surface area - mean size for non-porous particles of unknown shape: surface weighted mean of VSSA equivalent diameter
	Explanatory Videos: https://www.youtube.com/watch?v=F5mdtQ17JQU	

Nanoparticle Tracking Analysis (NTA)	This technique is based on the tracking of particle movement in liquid suspension using visualization of scattered laser light and subsequent analysis of Brownian diffusion motion that provides particle-by-particle size information and concentration. NTA is a particle characterization technique, which can measure the size and concentration of nanoparticles in liquid suspension. It simultaneously analyses particles on an individual basis, giving high resolution particle size distributions and direct measurements of the number concentration of particles within a sample, both with visual validation.	- high-resolution number-weighted particle size distribution - hydrodynamic spherical equivalent diameter of particles - particle number concentration in the suspension (concentration for specific size ranges can also be measured).
	Explanatory Videos: https://www.jove.com/t/61741/nanoparticle-tracking-analysis-gold-nanoparticles-aqueous-media	
Dynamic Light Scattering (DLS)	DLS is a technique that enables the measuring of the size of particles that are typically submicron and dispersed in liquid. It is a technique that can be used with a wide range of sample types such as inks, proteins and surfactants, that can use at their turn a range of solvents including both aqueous and organic solvents such as propanol. The size value of the nanoparticles is determined by measuring the translational diffusion coefficient of the nanoparticles in the liquid which is the velocity of Brownian motion.	- intensity weighted mean size value - particle size distribution
	Explanatory Videos: https://www.youtube.com/watch?v=ET6S03GeMKE	
Ultraviolet-visible spectrophotometry (UV/VIS)	UV-Vis spectroscopy is a simplified and inexpensive technique for nanomaterial characterization with non-trivial sample preparation that provides non-invasive and fast real-time screening evaluation of the nanomaterial properties. It can characterize the absorption, transmission, and reflectivity of a variety of materials, such as pigments, proteins, DNA, coatings, windows, filters, and many nanomaterials. It can provide qualitative results by revealing the identity of different components in a sample by their matching with the absorbance spectrum but can also be used quantitatively, as it can provide the concentration of a particular component in the sample. The working range is using a wavelength between 180 and 1100 nm and needs to be carried out in solutions.	- reveals the identity of different components in a sample by their matching with the absorbance spectrum - evaluation of the nanoparticle size (in real-time) and concentration - qualitative and quantitative results - it discerns between disperse or aggregated particles
	Explanatory Videos: https://www.jove.com/v/10204/ultraviolet-visible-uv-vis-spectroscopy, https://www.jove.com/t/61764/uv-vis-spectroscopic-characterization-nanomaterials-aqueous	
Laser desorption ionisation time-of-flight mass spectrometry (LDI-ToF-MS)	This technique is able of directly characterizing the organic capping and core composition of nanoparticles. Hereby, simultaneous bulk information is obtained from the core material composition as well as the type of capping agent (also referred to as coatings or surface ligands) present. This is especially enabled for metal nanoparticles which can absorb laser light energy of the commonly used laser wavelengths (e.g. Nd:YAG laser with 355 nm) and leads to analyte desorption and enhanced ionization. Thus, no additional organic matrix is	- chemical formula assignment for the capping agent - composition of the metal core - information on the bulk material, no single particle information

	needed to provide a source of ionization such as for matrix-assisted LDI (MALDI) that may introduce spectral and ionization interferences. The usage of the mass analyser ToF allows the parallel detection of all species, high-speed analysis with high transmission and theoretically unlimited mass range. The identification of the generated ions during the ionization process is based on the mass-to-charge (*m/z*) ratio resulting in a mass spectrum with signals for each ion. For the detected signals a molecular formula can be assigned.	
Single particle Inductively Coupled Plasma Mass Spectroscopy (spICP-MS)	SpICP-MS is an emergent ICPMS method for detecting, characterizing, and quantifying nanoparticles. Although the number of applications reported to date is limited, the relatively simple instrumental requirements, the low number concentration detection levels attainable, and the possibility to detect both the presence of dissolved and particulate forms of an element make this methodology very promising in the nanoscience related areas. SP-ICPMS takes advantage of the elemental technique of ICPMS but performing measurements on a "particle by particle" basis. spICP-MS can be considered one of the innovative and emerging analytical approaches demanded by the nano community. It is mainly used with suspensions of nanoparticles, in the size range from 1 to 100 nm. Although samples can be analysed in any aggregation state, liquid is the most common. Liquid samples are introduced into the ICPMS instrument by using a nebulization system, consisting of a nebulizer and a spray chamber, which produces an aerosol of polydisperse droplets. Once the droplets are into the plasma, solvent evaporates, forming solid particles, which in turn are vaporized and their elements atomized and ionized. Ions are extracted through the interface into the mass spectrometer, where they are separated according to their mass/charge ratio and detected.	- particle concentration in the suspension - mass of the element in the individually detected nanoparticles - particle size estimation based on the particle mass, composition, density and an assumed particle shape
	Explanatory Videos: https://www.youtube.com/watch?v=j1iJ7eMqFVQ	
Time-of-flight secondary ion mass spectrometry (ToF-SIMS)	TOF-SIMS is one of the few techniques that can provide specific compound identification of molecules on a surface. This technique focuses a pulsed beam of primary ions onto a sample surface, producing secondary ions in a sputtering process. Analysing these secondary ions allows identification of the atoms and molecules present on the surface of the particles, the absorbed molecules, and contaminants, such as oils, adhesives, etc., adsorbed on the surface. TOF-SIMS is a technique that detects all the elements in the periodic table, including hydrogen. TOF-SIMS can provide mass spectral image information in the XY dimension across a sample; and also, depth profile information on the Z dimension into a sample.	- detection of the chemical entity of the nanoparticle surface coating including chemical contaminants possible - detection and chemical discrimination between core and shell of particles possible in 3D depth profiles - nanoparticle size distribution patterns possible based on chemical information from 2D images (similar to TEM)
	Explanatory Videos: https://www.youtube.com/watch?v=ZoAUxsEBUnk	

Thermogravimetric Analysis - Fourier Transformed Infrared Spectroscopy, Gas Chromatography/ Mass Spectrometry (TG-IR-GC/MS)	It is a multihyphenated platform that incorporates physical and chemical property characterization. The sample is precisely weighed prior to analysis within the TGA. This mass is recorded in real time as the sample is slowly pyrolyzed under a nitrogen gas flow. During pyrolysis mass is lost through evolved gas of which circa 90% is transferred through a heated transfer line to a gas phase FTIR spectrometer which measures bond stretching, bending and twisting as infrared radiation is passed through the evolved gas. This data is then Fourier transformed to provide an interpretable spectrum. Following FTIR the evolved gas passes through another heated transfer line into the GC-MS loop. At a predetermined temperature, time or mass loss the GC-MS will do a full loop injection of this evolved gas. The complex mixture in this gas will then be separated as a property of thermal liability and hydrophobicity prior to ionization with electron ionization and detection using a single quadrupole mass spectrometer.	- organic polymer and additive identification
Two-Dimensional Liquid Chromatography Mass Spectrometry (2D-LC-MS)	The objective of the method is the detection and characterization of organic NPs in aqueous media. In this method, the organic NPs are size separated in the first dimension, a hydrodynamic chromatography column (HDC), of a 2D-LC-MS setup. Based on the HDC separation the particle size can be determined while the particle mass concentration may be determined using a UV-detector. Separated size groups are trapped in a sample loop and re-injected in an analytical liquid chromatography (LC) column that forms the second dimension. The sample loop is eluted with acetonitrile in which the organic NP micelles break up releasing the individual molecules comprising the particle. The individual compounds are analysed with high-resolution mass spectrometry (Orbitrap-MS) coupled on-line to the second-dimension analytical column. Based on the mass spectrum of the individual compounds the composition of the organic NPs may be determined.	- composition of the particle - composition of the particle corona
	Explanatory Videos: https://www.youtube.com/watch?v=UCA-5lLoGDQ	
Capillary Electrophoresis – Mass Spectrometry (CE-MS)	This technique is the result of hyphenating a separation technique based on the movement of ions under electrophoretic and/or electro-osmotic forces produced by the application of an electric field with a mass spectrometer. The technique is especially well suited for polar and ionic compounds in complex polar matrices.	- protein corona - metabolite corona
	Explanatory Videos: https://www.jove.com/t/61760/capillary-electrophoresis-mass-spectrometry-approaches-for	
Asymmetrical Flow Field-Flow Fractionation	Asymmetrical flow field-flow fractionation (AF4) separates nanomaterials in the size range of approx. 1-1000 nm according to their diffusion coefficient, respectively hydrodynamic size, based on the Stokes-Einstein-relationship. The fractionation takes place in a narrow ribbon-like channel, where a laminar flow is applied and is achieved by the application of	- hydrodynamic diameter - diffusion coefficient - additional information (e.g., size, shape, elemental composition) from on-line detectors

(AF4)	an adjustable separation force (cross flow) that acts perpendicular to the laminar flow. The cross flow exits the channel via a semi-permeable membrane at rhe channel bottom,	
	Explanatory Videos: https://www.youtube.com/watch?v=U8S6z-cvjko, https://www.jove.com/t/61757/asymmetrical-flow-field-flow-fractionation-for-sizing-gold	
Reactivity Assay	This technique is a simple colorimetric assay to detect the presence of NPs due to their catalytic activity. It detects the catalytic reactivity of NPs in aqueous media and can be used to screen for NPs catalytic surface activity in biological and environmental relevant samples. The measurement of the catalytic activity of NPs is based on the transfer mechanism between an organic dye, methylene blue (MB), and a reducing agent, sodium borohydride ($NaBH_4$), in the presence of NPs. When NPs are introduced to a MB-$NaBH_4$ solution, the NPs serve as a catalyst for reducing the dye-reductant agent pair by promoting the electron transfer between the dye and reductant. Reducing the dye from its oxidized state to its reduced state results in a colour change from blue (oxidized state) to colourless (reduced state) due to a change in absorbance properties between the oxidized and reduced dye molecules.	- catalytic reactivity (and derived from that nanoparticle concentration)

ANNEX I: *Complete List of Techniques included in the ACEnano's Knowledge Warehouse.*

TECHNIQUE	*UNIQUE URL / ID*
Assay-on-a-chip	https://ACEnano.douglasconnect.com/protocols/techniques/38/
Asymmetrical Flow Field-Flow Fractionation	https://ACEnano.douglasconnect.com/protocols/techniques/30/
Atomic Force Microscopy	https://ACEnano.douglasconnect.com/protocols/techniques/29/
Brunauer–Emmett–Teller analysis	https://ACEnano.douglasconnect.com/protocols/techniques/39/
Capillary Electrophoresis	https://ACEnano.douglasconnect.com/protocols/techniques/21/
Capillary Electrophoresis-Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/53/
Centrifugal Field-Flow Fractionation	https://ACEnano.douglasconnect.com/protocols/techniques/7/
Centrifugal Field-Flow Fractionation-MALS	https://ACEnano.douglasconnect.com/protocols/techniques/33/
Column test	https://ACEnano.douglasconnect.com/protocols/techniques/45/
Dialysis + ICP-MS	https://ACEnano.douglasconnect.com/protocols/techniques/35/
Disc centrifuge	https://ACEnano.douglasconnect.com/protocols/techniques/8/
Dye loaded field flow fractionation	https://ACEnano.douglasconnect.com/protocols/techniques/47/
Dynamic Light Scattering	https://ACEnano.douglasconnect.com/protocols/techniques/1/
Electrophoretic Light Scattering	https://ACEnano.douglasconnect.com/protocols/techniques/55/
Electrophoretic mobility	https://ACEnano.douglasconnect.com/protocols/techniques/22/
Energy Dispersive X-ray Spectroscopy	https://ACEnano.douglasconnect.com/protocols/techniques/56/
Energy Dispersive X-ray Spectroscopy in the SEM and TEM	https://ACEnano.douglasconnect.com/protocols/techniques/12/
Force tensiometry	https://ACEnano.douglasconnect.com/protocols/techniques/48/
Fourier Transform Infrared Spectroscopy	https://ACEnano.douglasconnect.com/protocols/techniques/58/
Full field transmission X-ray microscopy	https://ACEnano.douglasconnect.com/protocols/techniques/51/
Hydrophobic interaction chromatography	https://ACEnano.douglasconnect.com/protocols/techniques/46/
Inductively Coupled Plasma Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/9/
Ion-selective electrode	https://ACEnano.douglasconnect.com/protocols/techniques/37/
Laser Ablation Inductively Coupled Plasma Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/13/
Laser Desorption/Ionization Time of Flight Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/54/
Laser induced breakdown detection	https://ACEnano.douglasconnect.com/protocols/techniques/41/
MALS/SLS	https://ACEnano.douglasconnect.com/protocols/techniques/32/
Mastersizer	https://ACEnano.douglasconnect.com/protocols/techniques/26/
Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/16/
Nanoparticle Tracking Analysis	https://ACEnano.douglasconnect.com/protocols/techniques/5/
Nuclear magnetic resonance spectroscopy relaxation	https://ACEnano.douglasconnect.com/protocols/techniques/40/
Quartz crystal microbalance with dissipation monitoring	https://ACEnano.douglasconnect.com/protocols/techniques/19/
Raman spectroscopy	https://ACEnano.douglasconnect.com/protocols/techniques/15/
Scanning Electron Microscopy	https://ACEnano.douglasconnect.com/protocols/techniques/2/
Scanning Transmission Electron Microscope- Energy-dispersive X-ray spectroscopy	https://ACEnano.douglasconnect.com/protocols/techniques/17/
Scanning transmission X-ray microscopy	https://ACEnano.douglasconnect.com/protocols/techniques/52/
SEC/HDC/HIC	https://ACEnano.douglasconnect.com/protocols/techniques/31/
Single-Cell Single Particle Inductively Coupled Plasma Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/28/
Single Particle Inductively Coupled Plasma Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/27/

Single Particle Inductively Coupled Plasma Time-Of-Flight Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/11/
Small-Angle X-ray Scattering	https://ACEnano.douglasconnect.com/protocols/techniques/23/
Thermogravimetric Analysis	https://ACEnano.douglasconnect.com/protocols/techniques/57/
Thermogravimetric analysis coupled with Fourier Transform Infrared and Gas Chromatography/Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/20/
Time of flight secondary ion mass spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/10/
Time resolved Dynamic Light Scattering	https://ACEnano.douglasconnect.com/protocols/techniques/42/
Time resolved nanoparticle tracking analysis	https://ACEnano.douglasconnect.com/protocols/techniques/44/
Time resolved Single Particle Inductively Coupled Plasma Mass Spectrometry	https://ACEnano.douglasconnect.com/protocols/techniques/43/
Tip Enhanced Raman Scattering (nano-Raman)	https://ACEnano.douglasconnect.com/protocols/techniques/14/
Transmission Electron Microscopy	https://ACEnano.douglasconnect.com/protocols/techniques/4/
Transmission electron microscopy with electron energy loss spectroscopy	https://ACEnano.douglasconnect.com/protocols/techniques/49/
Ultracentrifugation + ICP-MS	https://ACEnano.douglasconnect.com/protocols/techniques/36/
Ultrafiltration + ICP-MS	https://ACEnano.douglasconnect.com/protocols/techniques/34/
Ultraviolet–visible spectroscopy	https://ACEnano.douglasconnect.com/protocols/techniques/3/

ANNEX IIa: *List of physico-chemical characterization techniques included in the Nano Method-Decision Tool linked to experts and facilities of the ACEnano project consortium that may provide support and guidance on nanomaterial characterisation.*

Contact Details: For more information about the contact details of the listed entities, visit http://www.acenano-project.eu/about-acenano/acenano-team.

	ACEnano's Project Partners									
	University of Birmingham	University of Vienna	Postnova Analytics GmbH	CSEM	Wageningen Food Safety Research	Perkin Elmer LAS	TOFWERK AG	Helmholtz-Zentrum für Umweltforschung	Malvern Panalytical Ltd	University of Oxford
Assay-on-a-chip				■						
Asymmetrical Flow Field-Flow Fractionation (AF4)	■	■	■	■				■		
Atomic Force Microscopy (AFM)	■			■	■					■
Brunauer–Emmett–Teller analysis (BET)	■	■								■
Capillary Electrophoresis-Mass Spectrometry (CE-MS)	■									
Dynamic Light Scattering (DLS)	■	■	■					■	■	■
Laser Desorption/Ionization Time of Flight Mass Spectr.(LDI-TOF-MS)								■		
Nanoparticle Tracking Analysis (NTA)	■				■				■	■
Nuclear magnetic resonance spectroscopy relaxation (NMR)	■									■
Scanning Electron Microscopy (SEM)	■			■	■			■		■
Single Particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS)	■	■	■		■			■		
Single Particle Inductively Coupled Plasma Time-Of-Flight Mass Spectrometry (spICP-TOFMS)		■					■	■		
Small-Angle X-ray Scattering (SAXS)				■					■	
Thermogravimetric analysis coupled with Fourier Transform Infrared and Gas Chromatography/Mass Spectrometry (TG-IR-GC/MS)	■					■				
Time of flight secondary ion mass spectrometry (TOF-SIMS)								■		
Transmission Electron Microscopy (TEM)	■			■	■					■
Ultraviolet–visible spectroscopy	■	■		■					■	■
X-Ray-Diffraction and Powder-X-Ray-Diffraction (XRD and PXDR)	■	■		■					■	■

ANNEX IIb: *Some of the European experts and facilities part of other European initiatives, infrastructures and/or projects that may provide support and guidance on nanomaterial characterisation.*

NNFA-EUROPE:

Website: https://www.nffa.eu/

Contact them by email: secretariat@nffa.eu

Main headquarters: AREA Science Park – Basovizza S.S. 14 Km 163.5, 34149 Trieste, Italy

JRC Nanobiotechnology Laboratory:

Website: https://ec.europa.eu/jrc/en/research-facility/nanobiotechnology-laboratory

CEITEC Nano:

Website: http://nano.ceitec.cz/

Contact them by email: info@ceitec.cz

Main headquarters: Brno, Czech Republic.

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