Characterisation of Nanomaterials in Complex Biological and Medical Samples: Advances and Challenges Imposed by Regulation

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National Measurement Laboratory (NML)

Measurement matters

CCQM Workshop on Particle Metrology, *25 - 27 October 2022*

Revision of European Commission Recommendation (2011/696/EU)

- The revised European Commission definition of the term "nanomaterial" was adopted on June 10 (https://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm)

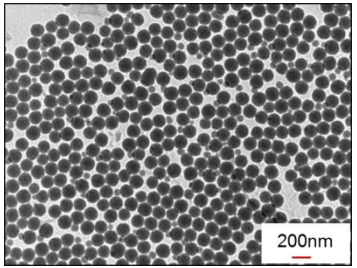
- The fundamental principle, namely that a nanomaterial is defined as a material where at least 50 % of the particles have at least one dimension < 100 nm was kept, but new recommendation:

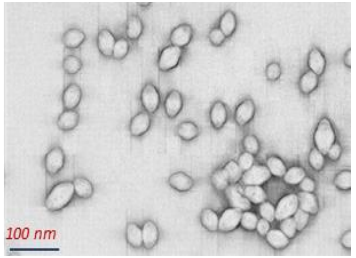
  > excludes non-solid (liquid and gaseous) particles e.g. micelles or nano-scale droplets in emulsions and sprays

  > states that additives or solvents (that can be separated without affecting the nanoparticle size distribution) are part of the nanomaterial but should no be taken into account when assessing whether a material is a nanomaterial

  > states that a material with a volume specific surface area less than 6 m^2/cm^3 should not be considered a nanomaterial.

  > replaced carbon nanotubes by a generic inclusion of nanorods/nanofibres and nanoplates





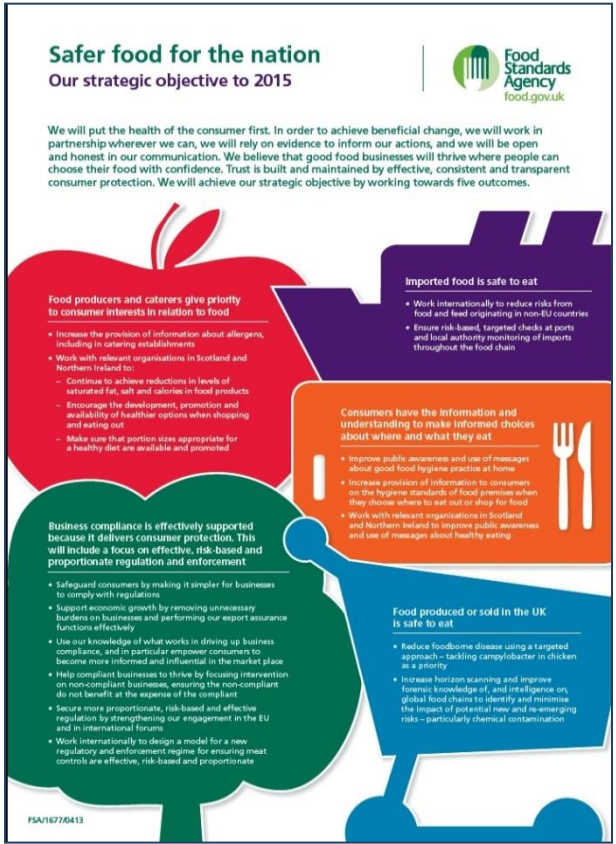
JRC Science for Policy Report: Identification of nanomaterials through measurements, **2019, DOI: 10.2760/053982** (Points to consider in the assessment of particulate materials according to 2011/696/EU)

Emerging nanoparticle regulation (nano as food additive)

- EU regulation 1169/2011 on food information for consumers
  - nanomaterials should be clearly indicated in the list of ingredients and names should be followed by (nano)

- Regulation **EC 2015/2283:** Foods consisting of ‘engineered nanomaterials’ are in the list of criterion to be considered for novel foods and therefore subjected to pre-market authorisation

- Regulation **EC 1935/2004** establishes that active and intelligent food contact materials and articles are included in its field of application. To ensure a high level of protection of human health and consumers' interests, food consisting of engineered nanomaterials should also be considered a novel food under this Regulation.



Cosmetics Regulation 1223/2009 and SCCS/1501/12 guidance - ZnO as approved additive

In case of combined use of Zinc Dioxide and Zinc Dioxide (nano), the sum shall not exceed the 25%

NM	Property to be measured (relevant to EU regulation)
Zinc oxide Max. concentration in final preparation: 25%*	purity 96≥% (impurities consisting only of carbon dioxide and water, whilst any other impurities are less than 1 % in total)
	crystal structure (wurtzite)
	median diameter of particle number size distributionD50 (50 % of the number below this diameter) > 30 nm and D1 (1 % below this size) > 20 nm
	Shape: physical appearance as clusters that are rod-like, star-like and/or isometric shapes
	water solubility < 50 mg/L
	Surface functionality (compound identification) Uncoated ZnO Coatings (triethoxycaprylylsilane, dimethicone, dimethoxydiphenylsilanetriethoxycaprylylsilane cross- polymer, or octyl triethoxy silane)

Other Regulations

- **Medical Devices Regulation (EU) 2017/745:**

There is scientific uncertainty about the risks and benefits of nanomaterials used for devices. In order to ensure a high level of health protection, free movement of goods and legal certainty for manufacturers, *it is necessary to introduce a uniform definition for nanomaterials based on Commission Recommendation 2011/696/EU, with the necessary flexibility to adapt that definition to scientific and technical progress and subsequent regulatory development at Union and international level.*

> In the design and manufacture of devices, manufacturers should take special care when using nanoparticles for which there is a high or medium potential for internal exposure. Such devices should be subject to the most stringent conformity assessment procedures.

Overview on analytical techniques for nanoparticle characterisation

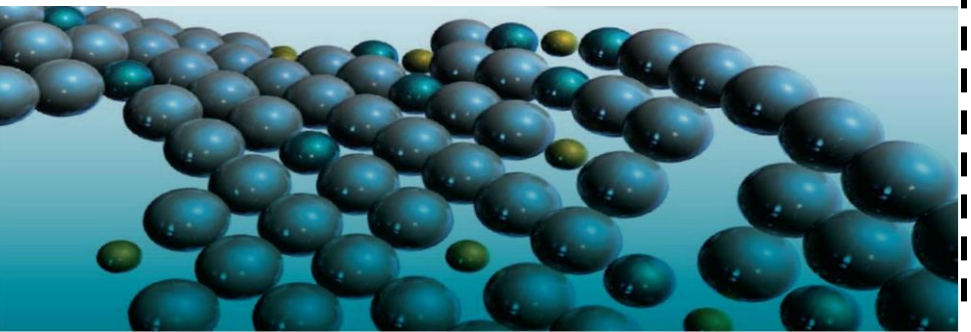
Nano-objects

- With metal cores (metal oxides, quantum dots, etc)

- Selected carbonaceous (C nanotubes)

- 1-100 nm including aggregated forms

- With a range of morphologies and geometries



Analytical techniques

Fractionation:
- Field flow fractionation (FFF)
- Hydrodynamic chromatography (HDC)
- Filtration and ultrafiltration
- Dialysis

Detection:
- (sp)ICP-MS, ICP-OES, AAS
- Electron microscopy (EM)
- Atomic force microscopy (AFM)
- Light scattering (DLS, MALS, MADLS)
- X-ray photoelectron spectroscopy (XPS)
- Brunauer-Emmett-Teller (BET)
- Small-angle X-ray Scattering (SAXS)
- X-ray diffraction (XRD)
- Particle tracking analysis (PTA)
- ESI Differential Mobility Analysis (ESI DMA)

Home message - Analytical drivers & challenges driven by EU definition/regulation

- **Number-based concentration**
  - particle counting techniques required
  - transforming mass to numbers

- **Measuring range down to a few nm**
  - Hardly available techniques to achieve accurate data for < 20 nm

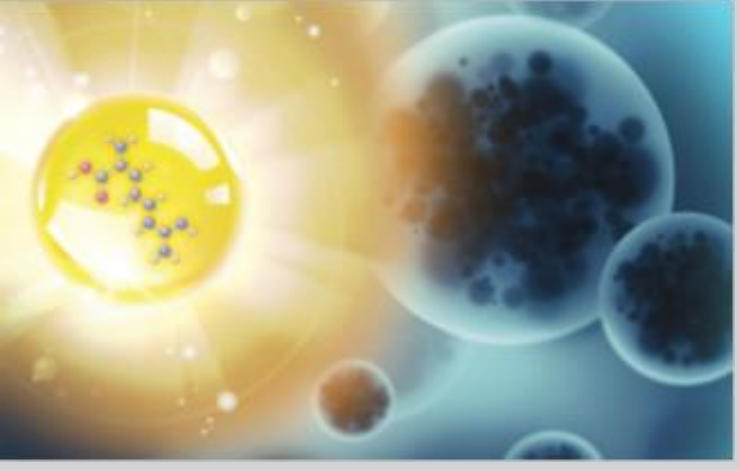
- **Possible NP changes during sample storage and preparation**
  - need for separation of primary particles from aggregates/agglomerates
  - de-agglomeration
  - development of on-line non denaturing fractionation techniques
  - Difficulty to achieve mass balance

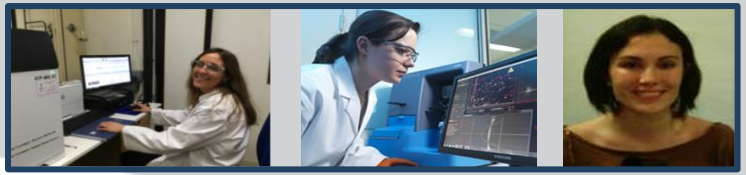
- **Lack of standards an reference materials for instrument calibration and method validation**

Need for validated accurate methods that:

> can help validate new and improved methods

> can be directly implemented by industry





Assessment of the safety of medical devices (e.g. containing antibacterial AgNPs) using spICP-MS and AF4-ICP-MS

AgNPs in antibacterial medical devices

- Antimicrobial wound dressings assist in removal of barriers to healing when clinical signs of infection are present, such as pain, redness and swelling (global market of **£208M)**
- PEG-coated Nano silver dressings have been shown to have prolonged antibacterial properties
- Manufacturers to comply with ISO/TR 10993-22:2017 - Guidance on nanomaterials and ISO 10993-1:2018 -- Evaluation and testing within a risk management process, and with many other regulations regarding nanoparticles in medical devices
- Question: How much AgNP and/or dissolved silver is released into the blood stream and/or accumulated in tissues?
- NP number concentration and size, as determined in the biological matrix, are essential

Workflow for material characterisation and safety assessment

NP Number concentration and size, ionic Ag

Wound treatment

(12 treated + 12 controls)

Blood (pre-dose, 1h, 3h, 24h, Day9, Day15, Day21, Day30)

Tissue pool per organ (spleen, liver, kidney, brain, testis)

TMAH digestion

spICP-MS

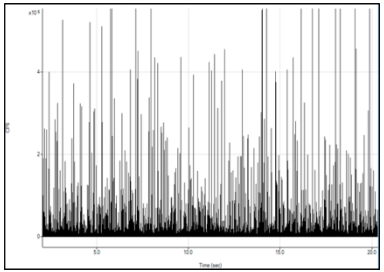
Leaching of AgNPs (ethanol, biological fluid)

AgNPs < 30 nm + ionic Ag?

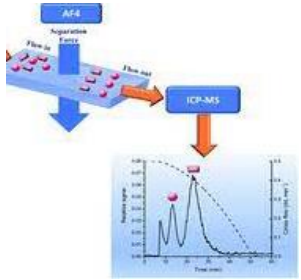
No

Yes

spICP-MS

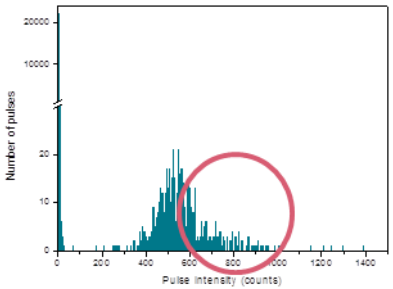


AF4-CP-MS

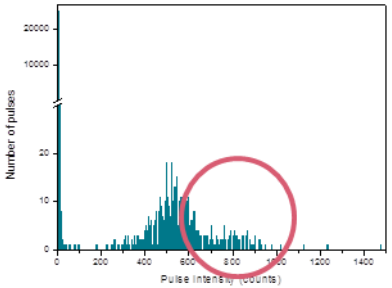


Animal study: Effect of the extraction/ digestion media on AgNP stability

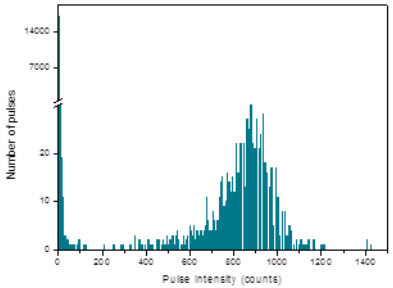
Ultrapure water



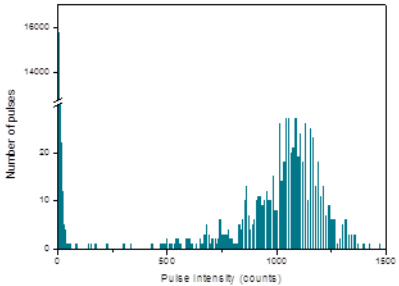
0.1% Triton X-100



1% TMAH; 0.1% Triton X-100



2.5% TMAH; 0.1% Triton X-100



Medium	Number of events
Ultrapure water	586 ± 28
0.1% Triton X-100	567 ± 20
1% TMAH, 0.1% Triton X-100	858 ± 21
2.5% TMAH, 0.1% Triton X-100	859 ± 18

NIST RM 8017
TEM: (74.6 ± 3.8) nm

I. Abad-Alvaro, *et al.*, ***J. Anal. At. Spectrom.***, 2021,**36**, 1180-1192, DOI: 10.1039/D1JA00068C

Animal study: Model samples for recovery & stability measurements by spICP-MS

Pig brain

Whole blood (from Seronorm)

+ 40 nm AgNPs

- NanoXact 40 nm PEG-AgNPs characterised for **number concentration by spICP-MS** (8900 ICP-MS):

  > Reference methodology based on DMF* method
  > Value compared with that using frequency method against NIST SRM 8017

- ✓ 95 ± 9% of Ag NPs spiked into tissue and blood were recovered following digestion with **TMAH** while maintaining the integrity of the Ag NPs

- ✓ Using the TMAH procedure blood digests were found stable for **8 days** whereas tissue digests were stable up to **30 days**

0.1g blood/homogenised tissue or 0.3g solid tissue

↓

15-25% (*v/v*) TMAH addition, 5min sonication, incubation (24 h in the dark)

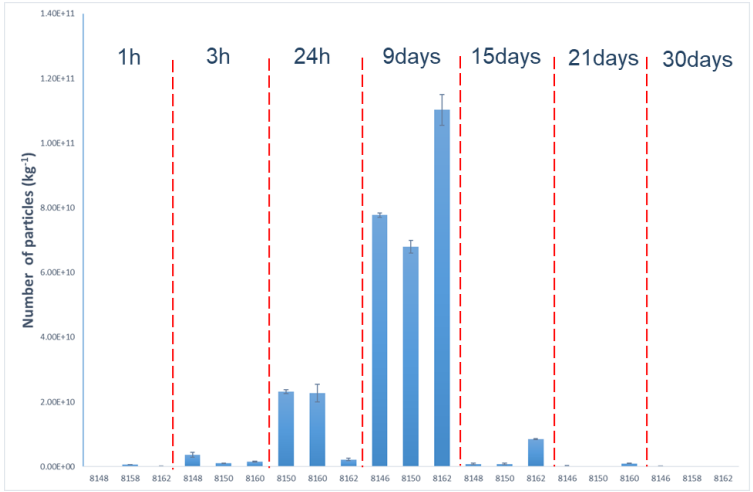
↓

Dilution with water (tissue) or 0.1% Triton-X (blood): 800-1100 particle events per minute

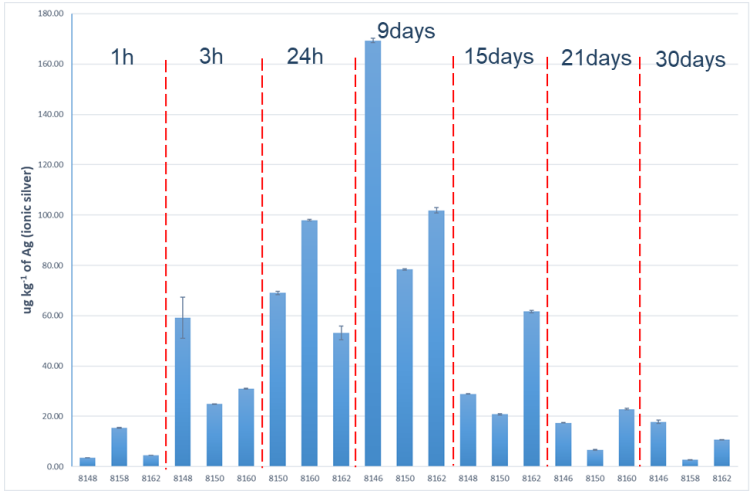
*S. Cuello, et. al., *J. Anal. At. Spectrom.*, 2020, DOI: 10.1039/c9ja00415g

Ionic Ag and AgNPs in rat blood by spICP-MS

Nanoparticles



Ionic Ag

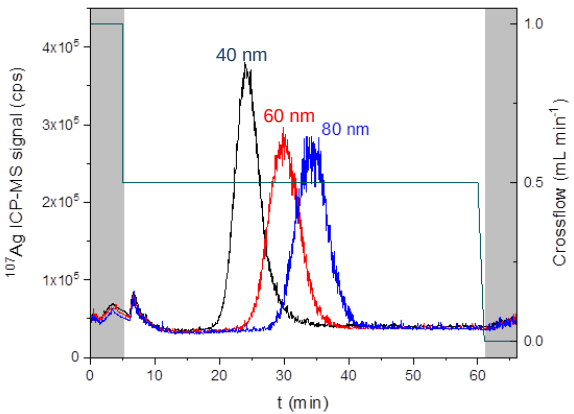


- ✓ Results for blood in treated samples show and increase in number of particles and ionic silver up to 9 days
- ✓ Particle with a diameter ranging from around 30 nm to 90 nm were detected. This diameter is based on a solid Ag sphere assumption.

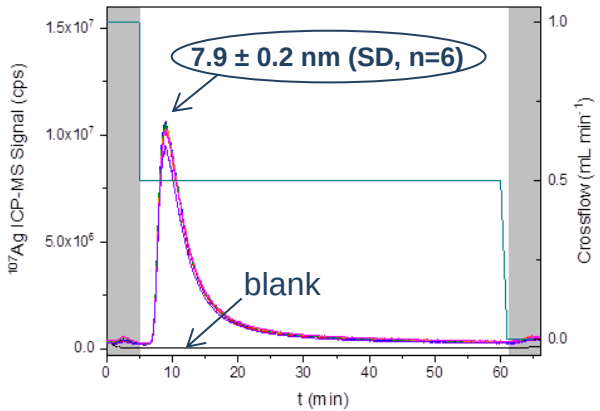
AgNPs characterisation in dressing leachates by AF4-ICP-MS: Towards meeting regulatory compliance

- ✓ **Size** (Elution time of AF4 size standards: 40 nm, 60 nm, 80 nm; FFF theory)
- ✓ **Number concentration** (Post-AF4 quantification of Ag mass fraction; conversion to number concentration considering size, bulk density of Ag (10.49 g cm^{-3}) and assuming spherical solid Ag)

Quality control AgNPs



Dressing leachate



Step	Time (min)	Flow profile	Crossflow (mL min^{-1})
Injection/focusing	5	injection flow 0.2 mL min^{-1}	1
Separation	55	constant	0.5
	1	linear decay	0.5 to 0
	7	constant	0

- ✓ ICP-MS (Agilent 8900)
- ✓ Isotopes: ^{107}Ag and ^{109}Ag, ^{103}Rh (IS)
- ✓ No gas mode
- ✓ AF4 eluate mixed with inorganic Ag standards in 20 µg kg^{-1} Rh, 1% (*v/v*) HNO_3

AF4 (Postnova); Carrier: 0.01% (w/v) SDS, pH 8, Membrane: 10 kDa Cellulose; Spacer: 350 µm

REMARKS

AgNPs and medical device safety:

- Workflows involving the characterisation of nanomaterials from toxicology studies usually involve a large number of samples that have to be processed and analysed within their stability window
- The availability of traceable methodology (DMF based) was found invaluable for the characterisation of commercial nanomaterials (more sample-like) that can be used as QCs and for transport efficiency determination in complex analysis workflows
- Higher throughput characterisation methods (e.g. spICP-MS) are preferred when larger number of samples with limited stability are involved. **But**, spICPMS size LODs many not be sufficient and the particle geometry may be required (for sizing).
- The complementary use of AF4-ICP-MS methodology may be found invaluable to obtain higher degree of information for complex samples containing AgNPs with size < 40 nm (in presence of ionic Ag)

LNE NANOTECH

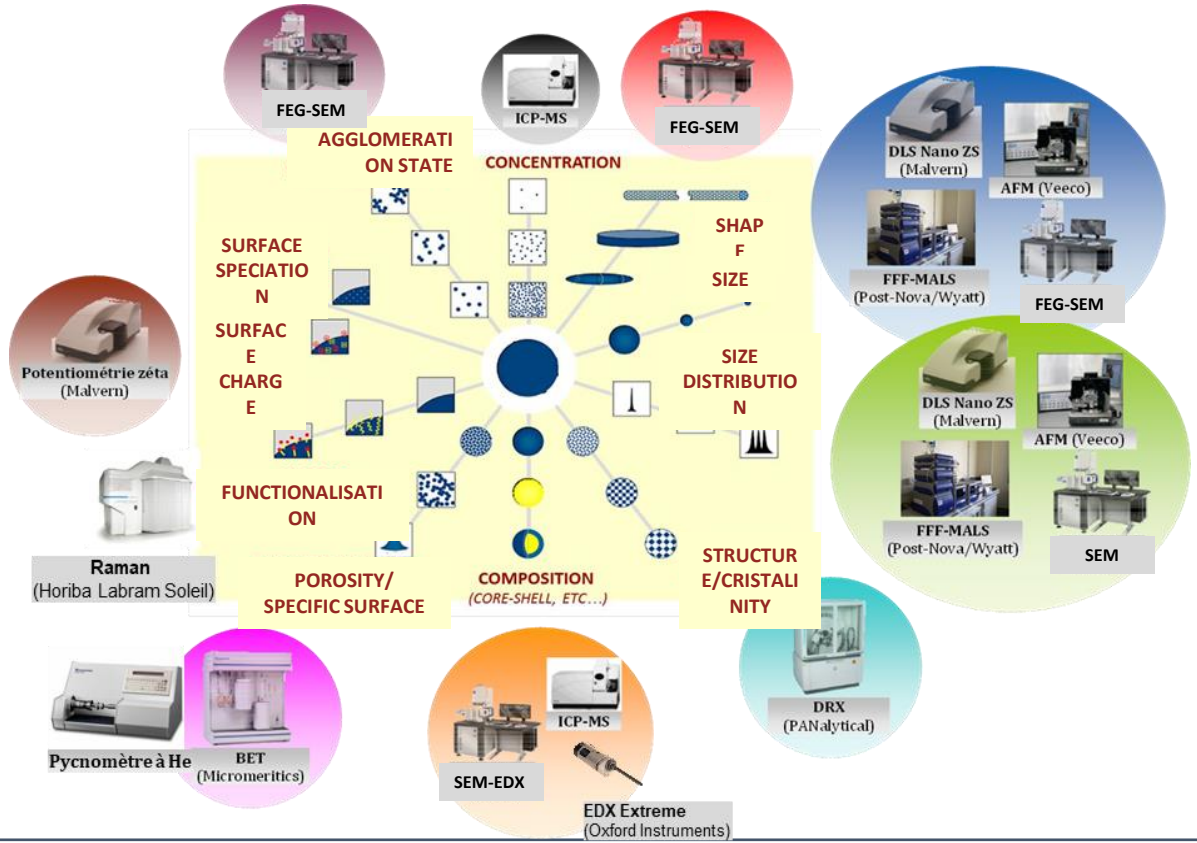
GENERAL CAPABILITIES FOR PARTICLE CHARACTERISATION

LNE PLATFORM:

... in particular to characterise

MOST of the Physico-chemical PROPERTIES, as well as their

MODIFICATION/ BIO-TRANSFORMATION IN A BIOLOGICAL ENVIRONMENT



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REGULATION FRAMEWORK

Implication in the development & VALIDATION of technical specifications (TS) / guidance documents on good practices

Nanomedicine:

TECHNICAL SPECIFICATION ASTM-E56:

Analysis of Liposomal Drug Formulations using Multidetector Asymmetrical-Flow Field-Flow Fractionation (AF4).

Analysis of LNP-RNA using Multidetector Asymmetrical-Flow Field-Flow Fractionation (AF4).

Guideline ASTM-E56

Characterization of Encapsulation, Extraction, and Analysis of RNA in Lipid Nanoparticle Formulations for Drug Delivery

Nano related topics:

CEN/TC 352 *Nanotechnologies - Guidance on the determination of aggregation and agglomeration state of nano-objects (2022 – 2025)*

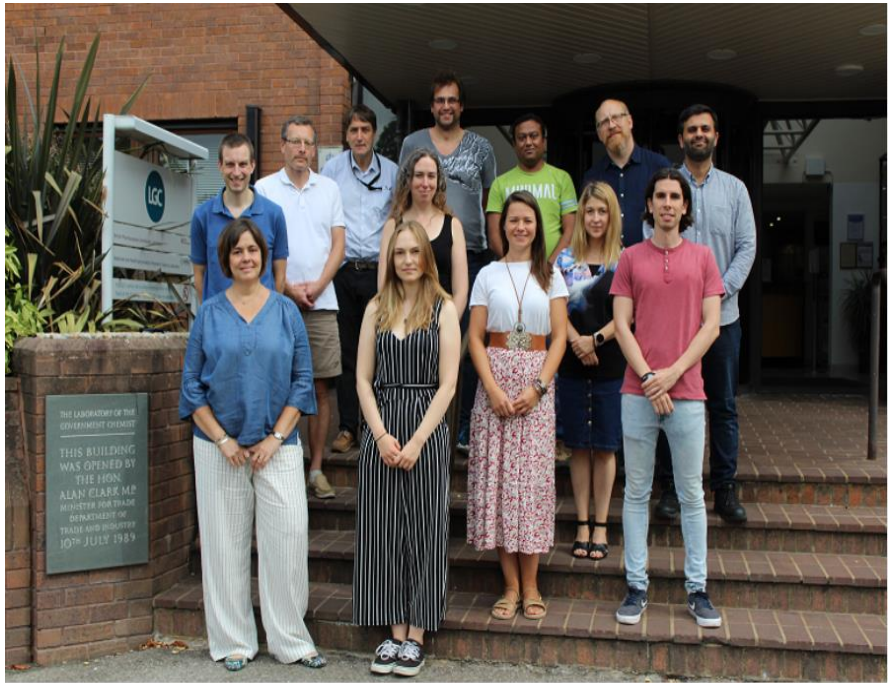
CEN/TC 352 *Guidelines for sample preparation, detection, identification and characterization by spICP-MS and EM-EDX of nano-objects in inorganic additives incorporated in food matrices*

Test Guidelines (TGs) developed with the OECD *(projects: measurement of nanopowders dustiness, specific surface, size distribution, solubility...)*

Harmonised test protocols in support of the regulatory framework with validation by inter-laboratory comparisons between expert laboratories

Acknowledgements

Inorganic Analysis Team, NML



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NanoChOp

INNANOPART

METVES II

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