The EU Definition of Nanomaterial – Potential Measurement Methodologies

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May 2012

Introduction

In October 2011 the European Commission published a definition of Nanomaterials[1]. This move followed more than six years of scientific consideration of the potential toxicological and environmental challenges posed by engineered nanomaterials.

The definition has these principal elements:

- Counting particles defines nanomaterials: The material is a nanomaterial if more than 50% of particles have at least one dimension between 1nm and 100nm.
- Alternatively, it is also a nanomaterial if it has a specific surface per unit volume of greater than 60 m^2/cm^3.
- There are specific inclusions such as graphene.
- Naturally occurring and incidental materials are included, as well as manufactured particles.
- Aggregates and agglomerates of such particles are included.

No measurement methods are specified; the recommendation is 'best available alternative methods should be applied[1]. This definition is not regulation, however its EU provenance informs its authority. For many regulators within the EU, this definition is the missing jigsaw piece to slot into potential regulation of publically-driven and government-derived legislation, covering nanomaterial matters from manufacture, labelling and handling, through transport and environmental fate. The FP7 project ObservatoryNANO describes current legislative work in their 4th report, April 2012[2].

The Particle Counting Characterisation Challenge

Given the definition that a nanomaterial contains more than 50% by particle number of material with at least one dimension in the range 1-100nm, there are a number of techniques that might be considered as contributing to the analysis of putative nanomaterials to help implement the proposed definition. While no one technique is likely to be able to address the whole range (especially with a requirement to count such material), a combination of such techniques would form the best available alternative and ensure a higher level of confidence in meeting this characterisation challenge.

The candidate techniques are grouped and their suitability reviewed below. A summary table is also provided (see **table 1**).

Single Particle Techniques

Given the EU definition explicitly requires counting of particles, methods which can

furnish such information would appear to be the only real option due to the known difficulty in obtaining true number distributions from bulk, ensemble techniques which generate a mass or volume distribution (discussed later).

1) Direct Imaging techniques

(Electron Microscopy, Scanning Probe Microscopy)

This category includes all scanning probe and electron microscopy. The direct imaging capability for particles down to 0.5nm ensures scanning probe and electron microscopy can capture information on particle size, shape and structure for the whole 1 – 100nm range. Additionally, some forms of microscopy can be used to retrieve information on material composition or chemistry. However, the primary drawback of microscopy is the time taken, especially when gathering statistically significant data, in sample analysis. Although automated detection software exists, it is still a time consuming process once sample preparation is factored in. Additionally, changes to the sample itself cannot be overlooked due to the preparatory steps required for this analysis. These forms of advanced microscopy have high capital and running costs and require operators to receive a high level of training. Despite these limitations it is still considered the most information-rich form of submicron characterisation.

2) Single Particle Counting techniques

(Flow cytometry, Optical single particle counters, Single Particle (sp) ICP-MS, Nanoparticle Tracking Analysis (NTA), Electrozone sensing (Coulter, IZON))

Single particle counting techniques for nanomaterial characterisation provide the most reliable methods for producing particle size distributions (PSDs) in a regular and routine use setting. As a class of technique they are fast and effective at providing PSDs and can analyse large numbers of particles to gather sample information. Distribution of single particle counting instruments is wide throughout analytical laboratories and the majority of systems have low running costs and high sample throughput. The direct number frequency distribution provided by single particle techniques clearly meets the requirements of the EC definition of nanomaterial in addition to the capability of certain techniques to work with fluorescently labelled particles. However, single particle techniques struggle to provide information on shape or structure often failing to resolve and enumerate constituent particles within agglomerates.

The majority of single particle techniques also have lower detection thresholds that fall short of the definition requirement. In many cases this limit lies between 10-50nm limiting the application of single particle techniques in fully satisfying the definition. A further criticism of some single particle techniques is the volume of a sample that is analysed is too small to provide a statistically strong PSD for the entire sample. However, the majority of single particle techniques process samples at a high through-rate allowing many subsections of a single sample to be characterised over a short period of time.

Ensemble Techniques

Such techniques measure a bulk material typically generating a single value which is an average of the population analysed. Accordingly, except through (uncertain) mathematical transformation from mass or intensity average information, no direct number count is available.

1) Light Scattering Ensemble Techniques

(Dynamic Light Scattering, Photon Correlation Spectroscopy, Multi-angle Laser Light Scattering)

Light scattering ensemble methods share many benefits of single particle counting techniques. These are relatively low cost and

easy to use techniques that can process samples quickly with little sample preparation. Light scattering techniques also have the advantage of a <1nm lower detection limit and produce reliable statistics based on a high number of particles measured in a single sample. However, ensemble light scattering techniques provide data on mean z-average particle size which provides no number information, a key requirement of the definition. (Note a number distribution can be calculated from the z-average but requires information on the material refractive index and generally considered to be inaccurate) Additionally, as a z-average mean, ensemble techniques are heavily biased to contaminants and aggregates making it questionable when analysing polydisperse and heterogeneous sample types.

2) Other Ensemble Techniques

(Analytical Disc Centrifugation)

Analytical Disc Centrifugation provides high resolution size distributions with excellent peak to peak resolving power covering a large size range (2nm - 50,000nm). The large number of particles measured also provides good statistical confidence in data generated from this technique. However, similarly to other ensemble techniques, disk centrifugation provides no direct number information. Additionally, knowledge of material density and morphology is required prior to analysis for accurate sizing.

Techniques which separate mixtures into component sub-population (e.g. FFF, SEC, CZE, etc) which can then be analysed by other techniques (e.g. Refractive index detectors, spectrophotometers, etc) have not been included here because they do not, in one sense, constitute analytical methods per se.

NTA in meeting the definition requirements

The criteria of the EU Nanomaterials definition are again summarised below and the following section lists these criteria and discusses the ability of NTA to meet these characterisation criteria.

Definition Summary

- 1-100nm external dimensions
- More than 50% of 1 – 100nm in number distribution
- Natural, incidental, manufactured
- Additional way to be classed as a nanomaterial is to demonstrate a specific surface per unit volume of greater than 60 m^2/cm^3
- Particles, agglomerates, aggregates

	Techniques	Number based Size Distribution	Coverage of target size range	Specific Surface per Unit	Structure	Aggregates	Shape
Direct Imaging	EM (SEM/TEM)	Yes	5-100nm	No	Yes	Yes	Yes
	Scanning Probe Microscopy	Yes	0.5-100nm	No	Yes	Yes	Yes
Single Particle Counting	Flow Cytometry	Yes	75-100nm	No	No	No	No
	Optical Single Particle Counters	Yes	75-100nm	No	No	No	No
	sp ICP-MS	No	10-100nm	Yes	No	No	No
	NTA	Yes	20-100nm	No	No	No	No
	Electrozone Sensing	Yes	50-100nm	No	No	No	No
Ensemble Techniques	DLS	No	1-100nm	No	No	No	No
	Multi-angle Light Scattering	No	1-100nm	No	No	No	No
	Disc Centrifugation	No	2-100nm	No	No	No	No

Table 1. The ability of available nanomaterial characterisation techniques to meet the requirements of the EC definition of nanomaterial

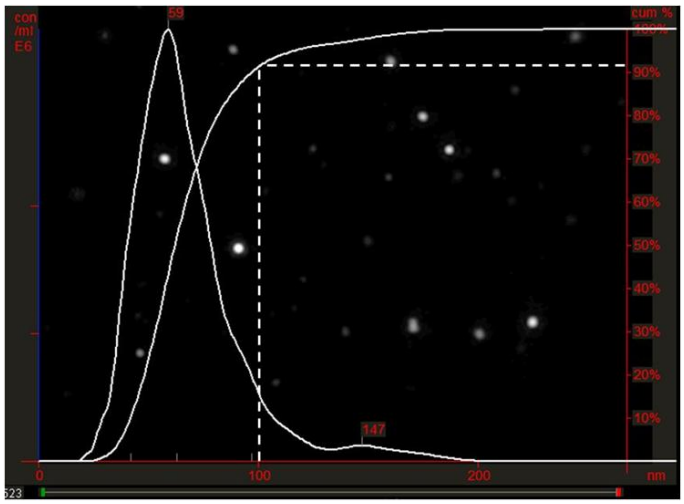

Figure 1. NTA number based PSD indicating over 90% of sample below 100nm

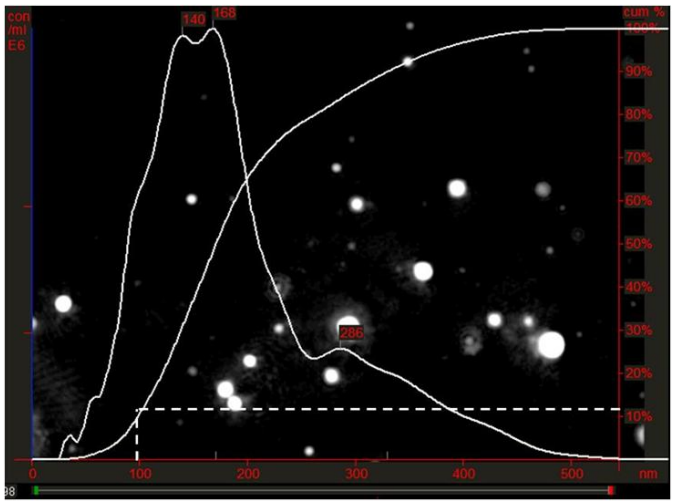

Figure 2. NTA number based PSD indicating only 11% of sample below 100nm

1 – 100nm size range

The range of particle sizes analysed by NTA depends on the material type. The lower size limit is defined by the particle size and refractive index. For particles with a high refractive index, such as metals or metal oxides, accurate determination of size can be achieved down to 15nm diameter. For lower refractive index particles, such as biological samples, the smallest detectable size might only be between 35nm and 40nm. The upper size limits are approached when Brownian motion of a particle becomes too limited to accurately track, typically 1–2µm diameter.

50% of particles by count between 1-100 nm

Although other light scattering techniques are well established, NTA is unique in its particle-by-particle approach. Each particle is simultaneously but separately visualised and tracked by a dedicated particle tracking image-analysis programme. The distance each particle moves is calculated and used to produce the individual particle diffusion coefficient (D_t) which identifies the particle hydrodynamic diameter (d). This information is brought together forming a number based particle size distribution of the sample. This approach allows the individual size of every particle that contributes to the PSD to be known. As a result the particle size and PSD produced by NTA is based on true particle data and does not suffer from the limitation of being an intensity weighted, z-average distribution. The particle size distribution profile obtained by NTA is a direct number/frequency distribution. This information can clearly highlight samples with over 50% of material below 100nm (see **figure 1 & 2**).

Natural, incidental, manufactured nanomaterial

NTA characterises nanomaterial based on size alone. This matches the requirements of the EC definition which focus on a number based size distribution requiring no separation between different nanomaterial populations based on chemical analysis.

However, NTA simultaneously measures particle size and relative particle scattering intensity. This allows heterogeneous particle mixtures to be resolved.

Particles, agglomerates, aggregates

NTA produces data on the hydrodynamic diameter (*d*) of material based on the Diffusion coefficient (D_t). The variable d is the diameter of a sphere which would diffuse through the same medium at an equal rate. NTA is unable to deliver information on structure or shape of nanomaterial but instead highlights the spherical equivalent diameter. A result of this approach is that NTA does not distinguish between aggregates or agglomerates. This is line with the EC definition that includes all particles, agglomerates and aggregates that fall within the 1 – 100nm size range.

NTA as a characterisation tool

The use of NTA as a nanomaterial characterisation tool has been well established in a number of varied applications. This includes production line quality control, biomedical development and nanotoxicology research. NTA data has correlated well with other characterisation techniques including Electron and Scanning probe microscopy, DLS, flow cytometry, spectroscopy and centrifugation (see **table 2**).

In a critical discussion of particle tracking techniques for the determination of size and concentration of nanoparticles in complex matrixes, Gallego-Urrea *et al*[19, 20] outlines the suitability of NTA. As an easy, low cost and rapid method, NTA provided high sensitivity particle number concentrations as well as high resolution in terms of size determination with a PSD not adversely influenced by the presence of larger particles or aggregates. However, Gallego-Urrea also highlighted areas NTA required development including statistical reliability and lower detection limits.

The value of NTA number based size distributions was also highlighted by Filipe *et al*[18] in a critical evaluation of NTA focused on protein aggregates. Filipe concluded that the PSD provided through number based analysis held greater relevance with polydisperse samples than intensity based Z-average data attained by DLS[18]. This view was further supported in an analysis of NTA and DLS characterisation of NIST Gold standard nanoparticles in biological medium[5]. This study concluded that both techniques attained size distributions closely matching the standard particle characterisation by NIST but suggested NTAs particle-by-particle approach generates a higher resolution of data.

NTA has been successfully established on a broad range of nanoscale material. An increasing number of studies exploiting NTA address the potential hazards of different metal species in a variety of cellular and aqueous systems. These studies included the characterisation of gold[21, 22] silver[23, 24] and copper and chrome oxide nanoparticles[25, 26].

In his assessment of the need for standardized methods and environmental monitoring programs for anthropogenic nanoparticles, Paterson reviewed the available techniques emphasising the critical need for methods capable of qualitatively and quantitatively measuring such pollutants. He issued a challenge to national and international regulatory and research agencies to help develop standard methods, quality assurance tools, and implement environmental monitoring programs for this class of pollutants citing NTA as being one such technique that could supply important information[27].

The application of NTA to analysing biological nanomaterial has been recently reviewed by Sokolova *et al*[17]. Exosomes were analysed using NTA, DLS and SEM in different experimental settings. Sokolova concluded that a combination of NTA and SEM provided the most appropriate technique for determination of exosome size and integrity specifically highlighting the strength of this approach in analysing polydisperse samples.

	Electron Microscopy	Scanning Probe Microscopy	DLS	Spectroscopy	Centrifugation	Static Light Scattering	Nanomaterial
Tan B, Lee J-Y, Cooper AI[3]	SEM						Polymer
Trushkevych O, *et al*[4]	SEM, TEM	AFM	X	Absorption, Resonant Ramen			CNT
Montes-Burgos I, *et al*[5]			X				Gold
Holmberg J, *et al*[6]			X				TiO2
Moser M[7]	TEM		X		X	X	Viral vaccine
Marsh D H, *et al*[8]	TEM		X	UV-Vis absorption			Gold NPs
Colognato R, *et al*[9]	SEM						Cobalt NPs
Tran Le Thu, *et al*[10]			X				Casein micellar
Kendall K, Kosseva MR[11]			X				Iron hydroxide
Barcikowski, *et al*[12]	TEM		X				Silver
Ben-Moshe T, *et al*[13]	TEM						Copper
Kendall K, *et al*[14]	TEM						Hematite
Jones-Lepp T, *et al*[15]	TEM	AFM	X	UV-Vis absorption			Metal based
Klein, *et al*[16]	SEM, TEM		X				Silver NP
Sokolov, *et al*[17]	SEM		X				Exosomes
Filipe, *et al*[18]			X				Protein Aggregates/ Polystyrene

Table 2. Validation of NTA using a range of characterisation techniques

Framework for use

Given current technology, gathering a definitive understanding of nanoparticle samples requires multiple approaches.

The sized based detection limits of NTA incorporate the majority of the EC definition size range for nanomaterial. This highlights NanoSight as a valuable tool in preliminary and regular screening and assessment of potential nanomaterial substances.

This view was supported by Jones *et al*[15] concluding that no one testing technique can provide the desired measurement information for screening tests. Jones recommends employing complimentary microscopy-based and light scattering methods highlighting NTA as a good candidate to be supported by SEM, TEM or AFM[15].

Near-Future Development

Whilst NTA provides robust results in the hands of an experienced user, work continues to enable high levels of reproducibility from lightly trained and occasional-use operatives. This work has these elements:

- Critical consideration of the elements making up the error budget for NTA systems.
- Definition of confidence limits for acceptance of results.
- Provision of smart-automation for the few remaining parameters that users set. This is aimed at taking any subjective input by removing choice, with the software mimicking an expert user.
- Methodologies to close the bottom-end gap between NTA's current lower limit and the lower bound of the definition, at 1nm.

Conclusion

There is no single solution for particle characterisation in this sub-visible size range which completely answers the number count criteria set by the EU Nanomaterials definition.

Electron microscopy remains the gold standard, however the time taken to gather statistically significant data is prohibitive in routine analyses.

Ensemble techniques are just that, and cannot address counting requirements.

Most of the available single particle counting techniques have lower limits of detection above the top bound of the definition's 1-100 nm range. Nanoparticle Tracking Analysis (NTA) comes closest, with its lower limit between approximately 10 and 40nm, dependent on the material.

It is proposed that the best methodology currently available is a combination of routine NTA and occasional electron microscopy. NTA can provide count and particle size distribution, and complementary occasional use of electron microscopy can inform the bottom end of the distribution.

References:

1. The European Commission (2011) Commission Recommendation of 18 October 2011 on the definition of nanomaterial. Official Journal of the European Union (2011/696/EU). http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:275:0038:0040:EN:PDF.
2. Mantovani E, Porcari A, Morrison M D, Geertsma R E (2012) Developments in Nanotechnologies Regulation and Standards 2021 - Report of the Observatory Nano. April 2012. www.observatorynano.eu.
3. Tan B, Lee J-Y, Cooper A I (2006) Ionic hydrocarbon surfactants for emulsification and dispersion polymerization in supercritical CO2. Macromolecules 2006; 39(22): 7471–7473.
4. Trushkevych O, Collings N, Hasan T, *et al*. (2008) Characterization of carbon nanotube–thermotropic nematic liquid crystal composites. J Phys D: Appl Phys 2008; 41: 125106–125117.
5. Montes-Burgos I, Walczyk D, Hole P, *et al*. Characterisation of nanoparticle size and state prior to nanotoxicological studies. Journal of Nanoparticle Research. 2011; 7(1): 195-196.
6. Holmberg J, Urrea G J, Hassellöv M, *et al*. (2008) Synthesis, characterisation and aggregation behaviour of TiO2 nanoparticles in natural aquatic media. Nanoparticles in the Environment, Birmingham, September 2008.
7. Moser M (2008) Emerging analytical techniques to characterize vaccines. Proc Intl Conf Vaccines Europe, Brussels, December 2008.
8. Marsh D H, Rance G A, Higgins S J, Richards O J, Khlobystov A N (2008) Direct Space, Real-Time Observation of Gold Nanoparticle Growth & Aggregation Processes, The 82nd ACS Colloid & Surface Science, Raleigh, NC.
9. Colognato R, Bonelli A, Ponti J, Farina M, Bergamaschi E, Sabbioni E, Migliore L (2008) "Comparative genotoxicity of cobalt nanoparticles and ions on human peripheral leukocytes in vitro", Mutagenesis Advance Access, published online May 25, 2008. and Mutagenesis 2008; 23(5):377-382.
10. Tran Le Thu, Saveyn P, Dinh Hoa H, Van der Meeren P (2008) Determination of heat-induced effects on the particle size distribution of casein micelles by dynamic light scattering and nanoparticle tracking analysis, International Dairy Journal. 2008; 18(12):1090-1096.
11. Kendall K, Kosseva M R (2006) Nanoparticle aggregation influenced by magnetic fields, Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2006; 286(1-3):112-116.
12. Barcikowski S, Menendez-Manjon A, Chichkov B, Brikas M, Raciukaitis G (2007) Generation of nanoparticle colloids by picosecond and femtosecond laser ablations in liquid flow, Appl. Phys. Lett, NANOSCALE SCIENCE AND DESIGN. 2007; 91(8): 083113.
13. Ben-Moshe T, Dror I, Berkowitz B (2009) Oxidation of organic pollutants in aqueous solutions by nanosized copper oxide catalysts. Applied Catalysis B: Environmental. 2009; 85:207–211

14. Kendal K, Dhir A, Du S (2009) A new measure of molecular attractions between nanoparticles near kT adhesion energy. Nanotechnology. 2009; 20: 275701
15. Jones-Lepp T, Rogers K, Snell R (2011) Laser Detection of Nanoparticles In the Environment APM 31. EPA/600/R-11/097 September 2011
16. Klein C L, Comero S, Stahlmecke B, Romazanov J, Kuhlbusch T A J *et al* (2011) NM-Series of Representative Manufactured Nanomaterials, NM-300 Silver, Characterisation, Stability, Homogeneity. JRC Scientific and Technical Reports. DOI 10.2788/23079
17. Sokolovaa V, Ludwigb A, Hornungb S, Rotana O, Hornb PA, Epplea M, Giebelb B (2011) Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids and Surfaces B: Biointerfaces. 2011; 87: 146– 150
18. Filipe V, Hawe A, Jiskoot W (2010) Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. Pharmaceutical Research. 2010; 27: 796-810
19. Gallego-Urrea J A., Tuoriniemi J and Hassellöv M (2011) Applications of particle-tracking analysis to the determination of size distributions and concentrations of nanoparticles in environmental, biological and food samples, TrAC Trends in Analytical Chemistry, Volume 30, Issue 3, Pages 473-483
20. Gallego-Urrea J A., Tuoriniemi J, Pallander T, Hassellöv M (2010) Measurements of nanoparticle number concentrations and size distributions in contrasting aquatic environments using nanoparticle tracking analysis , Environmental Chemistry. 2010; 7(1):67–81
21. Ponti J, Colognato R, Franchini F, Gioria S, Simonelli F, Abbas K, Uboldi C, Kirkpatrick J, Holzwarth U, Rossi F (2009) A quantitative in vitro approach to study the intracellular fate of gold nanoparticles: from synthesis to cytotoxicity, Nanotoxicology, 22 July 2009, DOI: 10.1080/17435390903056384
22. Gosens I, Post J A, de la Fonteyne L J J, Jansen E H J M, Geus J W, Cassee F R, de Jong W H (2010) Impact of agglomeration state of nano- and submicron sized gold particles on pulmonary inflammation, Particle and Fibre Toxicology. 2010; 7:37 DOI:10.1186/1743-8977-7-37
23. MacCuspie R I, Rogers K, Patra M, Suo Z, Allen A J., Martin M N and Hackley V A (2011) Challenges for physical characterization of silver nanoparticles under pristine and environmentally relevant conditions, J. Environ. Monit., 2011. Advance Article, DOI: 10.1039/C1EM10024F
24. Bouwmeester H, Poortman J, Peters R J., Wijma E, Kramer E, Makama S, Puspitaninganindita K, Marvin H J P, Peijnenburg A M C and Hendriksen PJM (2011) Characterization of Translocation of Silver Nanoparticles and Effects on Whole-Genome Gene Expression Using an In Vitro Intestinal Epithelium Co-Culture Model, ACS Nano, Just Accepted Manuscript, DOI: 10.1021/nn2007145
25. Studer A M, Limbach L K, Van Duc L, Krumeich F, Athanassiou E K, Gerber L C, Moch H, Stark W J (2010) Nanoparticle cytotoxicity depends on intracellular solubility: Comparison of stabilized copper metal and degradable copper oxide nanoparticles, Toxicology Letters. 2010; 197(3):169-174
26. Khatoon I, Vajpayee P, Singh G, Pandey A K, Dhawan A, Gupta K C, Shanker R (2011) Determination of Internalization of Chromium Oxide Nano-Particles in Escherichia coli by Flow Cytometry, Journal of Biomedical Nanotechnology. 2011; 7(1):168-169(2)
27. Paterson G, Macken A, Thomas K V (2011) The need for standardized methods and environmental monitoring programs for anthropogenic nanoparticles, Anal. Methods, 2011, Advance Article, DOI: 10.1039/C1AY05157A