Final Publishable JRP Summary for JRP NEW03 Nano ChOp Chemical and Optical Characterisation of Nanomaterials in Biological Systems

Overview

Nanomaterials are being used widely, and by an ever growing number of European industries to develop the next generation of high-performance products. However, the properties of nanomaterials are not always well understood, and there are concerns about their potential toxicity. This project developed reference nanomaterials and measurement techniques to describe their chemical, physical and optical properties in biological systems. These materials and techniques are being used to better understand the properties of nanomaterials to assess their health risks, but also to support the effective use of nanomaterials in a wider range of European industries and products.

Need for the project

Nanomaterials are products that include structures smaller than 100 nanometres in length. Their small size, large surface area, and their quantum effects give them with a range of desirable properties such as increased strength, high elasticity, electrical conductivity, or radiation resistance. These properties are being used to develop the next generation of materials and products, and nanomaterials are used in an increasingly broad range of industries, including electronics, healthcare, cosmetics and clothing. It is estimated that nanomaterials are used in over 1300 products in a global market currently worth €9.6 billion*. However, although the properties of nanomaterials are attractive, they are not always well understood, and there are concerns about their potential toxic effects. To ensure the future competitive success of the wide range of European industries that use nanomaterials, methods are needed to accurately measure their properties in order to understand their effects on health.

The techniques developed need to be traceable to agreed reference systems, to guarantee the accuracy and repeatability of their results, but before this project there were no established reference nanomaterials to ensure comparability of toxicity studies and materials testing. Nanomaterials are also typically tested in non-biological media, but when nanomaterials interact with biological systems their properties can change significantly. Before this project there were no robust methods to describe nanomaterial properties in biological media such as cell cultures. New techniques were urgently needed to measure the physical, chemical and optical properties of nanomaterials in biological systems for safety and risk assessments.

**McWilliams G A A 2010 BCC Market Research Reports (NAN031D) p 1–276*

Scientific and technical objectives

The following five objectives were identified to achieve the overall goal of developing methods to measure the properties of nanomaterials in biological systems. Objective 1 developed a range of reference nanomaterials, and cell-based models that represent biological systems. These reference materials and biological systems were then used to develop nanomaterial measurement methods, including: size and chemical properties in a serum-based biological system (objective 2); real-time measurement of physical and chemical properties in a cell-based biological system (objective 3); optical properties of fluorescent nanomaterials in a serum-based system (objective 4); and fluorescent properties of nanomaterials attached to antibodies in biotechnology procedures (objective 5).

1. To produce a series of nanomaterials (for example, inorganic oxides and quantum dots) that are characterised in their native form for size, surface charge and fluorescence. To characterise a suitable cell-based model(s) for its application as a test system(s) for the interaction of nanomaterials with biological systems.

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2. To validate the use of a range of physical and chemical techniques for measuring the size and chemical composition of nanomaterials in a serum based biological system.
3. To develop methods for the simultaneous characterisation of physical and chemical composition of nanomaterials in cell based biological systems.
4. To develop traceable methods for the characterisation of 'bulk' optical properties of fluorescent nanomaterials, in particular quantum yield, absorption coefficient and corrected emission spectra.
5. To develop measurement techniques for biotechnology using fluorescent nanomaterials.

Results

1. To produce a series of reference nanomaterials. To characterise a suitable cell-based model(s) as a test system(s) for the interaction of nanomaterials with biological systems.

Reference nanomaterials and cell-based test systems were required as a first step, from which techniques could then be developed to measure the properties of the nanomaterials in the test biological systems. Manufactured silica nanoparticles were the focus of this research as they are commonly used as food additives. However, nanomaterials with ideal properties (e.g. spherical, monodisperse) were developed, rather than the typical nanomaterials used in everyday life, as this project represents the first step in addressing the measurement challenges of nanoparticle properties in biological systems.

A series of nanomaterials were produced in suspension, including plain colloidal silica and NH_3-modified colloidal silica (i.e. inorganic oxides for physical and chemical characterisation), fluorescently labelled inorganic oxides (e.g. fluorescently labelled silica for monitoring within biological cells), and quantum dots (for optical characterisation).

Homogeneity and stability studies were completed for each of the nanomaterials in suspension, and material information sheets were produced for each summarising their main characteristics. Protocols for dispersion of the nanomaterials in water, buffered aqueous solutions (e.g. Tris-HCl buffer solutions) and biologically relevant media were developed. The liver cell line HepG2 was selected as a suitable cell model to be exposed to the nanoparticles to characterise their properties in biological systems, and toxicity studies were undertaken. These showed that whilst quantum dots had no significant toxic effect, plain colloidal silica nanoparticles exhibited dose-response toxic properties, strongly indicative of a biological effect on the liver cells.

The project successfully achieved the objective as a series of reference nanomaterials were produced, appropriate cell-based test systems were identified, allowing the reference nanoparticles to be characterised in both their native form as well as within biological systems. The reference nanomaterials were then used by project partners for method development, validation and instrument calibration to deliver objectives 2-5.

2. To validate the use of a range of physical and chemical techniques for measuring the size and chemical composition of nanomaterials in a serum based biological system.

Multiple physical and chemical methodologies were developed and validated. Then, through a series of inter-laboratory studies, the size, size distribution, surface charge, concentration (chemical composition) and agglomeration of the plain colloidal silica and NH_3-modified colloidal silica reference nanomaterials were characterised in water, buffered aqueous solutions (e.g. Tris-HCl buffer solutions) and biological serum. The methods used included small-angle X-ray scattering (SAXS), field flow fractionation coupled with multi-angle light scattering (FFF/MALS), differential scanning calorimetry (DCS), dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA).

The objective was successfully achieved, as the effect of the biological medium and fluorescent staining on size measurements, and the influence of particle agglomeration (caused by the biological media) on number-based concentration measurements has been evaluated for the first time. In addition, the first method for determining the concentration of silica nanoparticles in a biological matrix using NTA has been developed. The potential benefits and drawbacks of the different techniques used (SAXS, FFF/MALS, DCS, DLS and NTA) were also summarised and published (see Sikora *et al.* 2015 in the list of publications).

3. To develop methods for the simultaneous characterisation of physical and chemical composition of nanomaterials in cell based biological systems.

To address the need for real-time physical and chemical characterisation of nanomaterials in biological samples, a novel methodology combining FFF with inductively coupled plasma mass spectrometry (ICP-MS) was developed and validated to measure chemical composition and size. The project produced, for the first time, silica nanoparticles isotopically enriched with ^{29}Si, and used them for accurate size-specific isotope dilution quantification (SI traceable) in aqueous suspensions using FFF-ICP-MS, with an expanded uncertainty of approximately 3.9%. The objective was achieved, and this measurement capability is invaluable for future chemical characterisation of reference nanomaterials. The know-how gathered on the method development and validation was also included in the BSI Publicly Available Specification (PAS) 139 guidelines (Detection and characterisation of nanomaterials in biological samples).

4. To develop traceable methods for the characterisation of 'bulk' optical properties of fluorescent nanomaterials, in particular quantum yield, absorption coefficient and corrected emission spectra.

Fluorescent nanomaterials can be used to develop easy-to-use techniques for measuring the number of molecules in a sample (i.e. by binding to them and being revealed under UV illumination). The project developed a validated protocol for the SI traceable determination of relative and absolute fluorescence quantum yield of the fluorescent nanomaterials in aqueous media and biological serum. Traceable spectroscopic methods were also developed and validated for determining the parameters affecting the signalling behaviour of fluorescent nanomaterials, such as the number of proteins adsorbed onto the nanomaterials in biological systems.

With these methods the project successfully achieved its objective. The protocol for the traceable determination of relative and absolute quantum yields has been published as a journal paper and made available to laboratories working in the field of quantum yield analysis.

5. To develop measurement techniques for biotechnology using fluorescent nanomaterials.

Fluorescent nanomaterials can be attached to antibodies that bind with specific target molecules (such as those produced by tumour cells), to indicate the presence, and importantly, the quantity of the target molecule in a sample. To better understand how the properties of antibody-bound nanoparticles influence their performance in biotechnology procedures, the fluorescent nanoparticles produced in objective 1 were integrated into an Interleukin 6 (IL-6) antibody procedure. This procedure was then used to successfully demonstrate the use of disc centrifugation for determining the relative quantities of agglomerated antibody conjugated nanoparticle.

The objective was successfully achieved, as suitable levels of accuracy were obtained for the procedure to be appropriate for validation of measurements in biotechnology laboratory processes. In addition, the uncertainty data generated by this research highlighted ways that manufacturers of biotechnology procedures can improve the performance of quantitative lateral flow assays.

Actual and potential impact

Dissemination of results

To promote the uptake of the reference materials and techniques developed, project results were shared broadly with scientific and industrial end-users. 11 papers were published in international scientific journals (listed in the next section). The project was publicised with an article '*Nanotechnology: The Big Challenge Behind the Characterization of the Small*', published on both the UK Nano Knowledge Transfer Network website, and the Drug Discovery & Development website (the latter reaches approximately 35-50k readers from the pharmaceutical industry, academia, manufacturers of instruments and nanomaterials). 47 presentations were given at international symposiums, including Nanosafety 2013 and 2014. The research consortium has also been active in organising and participating in workshop and training events with the end user community, including a Nanoparticle Workshop in April 2015, with approximately 100 end users, predominantly from higher education and public research organisations.

Impact on standardisation

The project has had significant impact on standards for assessing nanoparticle properties and safety. Examples include: LGC's contribution to the production of BSI guidelines (PAS 139) on the detection and characterisation of nanomaterials in biological samples (related to the international standard ISO/TC 229). BAM's contribution to the production of a standard guide for fluorescence measurements [ASTM E2719-09(2014)]. JRC's input into the draft documentary standard ISO 22412 "Particle size analysis-dynamic light scattering" (related to ISO/TC 24), and their input into vocabulary/terminology for ISO/TC 229 documents on nanotechnologies [e.g. quantum phenomena in nanotechnology (DTS 80004-12) and Plain Language Guide to vocabulary (ISO/TR 18401)].

Early impact on industry

Project outputs have already been shared with industry, and have been used to improve products and services: Nanomaterials from microParticles GmbH, PlasmaChem, Colorobbia and CAN were tested during work for objective 1. Results from testing were fed back to these companies, particularly on the stability, homogeneity, polydispersity and other important production quality parameters. This feedback, and knowledge acquired during the project, will be used to manufacture higher-performance nanomaterials for academic and industrial users, and is being used in the current EURAMET project Innanopart to develop new methods to characterise nanomaterial properties for industry. Insights developed from the production of reference nanomaterials were also shared with partner National Measurement Institutes, and guidance on the in-house preparation of quality control nanomaterials has been shared with reference material producers.

Malvern Instruments Ltd (a manufacturer of laboratory analytical equipment) has adopted the method developed in objective 2 for evaluating the performance of nanoparticle tracking analysis (NTA), to measure nanoparticle concentrations and sizes. Malvern's NTA output in terms of number based concentration offers great potential to assess if materials meet the European Commission's definition of a nanoparticle, necessary for determining whether or not the material will be subject to European safety regulations. Additionally, feedback was provided to Malvern Instruments on recommended improvements to their NTA software. The software has since been upgraded, and now allows users to study individual nanoparticle populations, and to resolve them within high serum concentrations, as required by nanotoxicology tests. In recognition of the project's contributions, Malvern Instruments have published a case study on their website describing the benefits of Nano ChOp results for their business. Following from the successful contributions to Malvern Instruments, CPS Instruments (another manufacturer of laboratory equipment) has entered into discussion with Nano ChOp researchers to explore ways to improve their centrifugal liquid sedimentation instrumentation software, and their related calibration approaches for nanomaterial characterisation.

As part of objective 3, the project evaluated equipment from Postnova Analytics (a developer of nanoparticle detection systems). Feedback was given to Postnova on their field flow fractionation detection modules, and as a consequence the company has upgraded a number of their products, which will enable academic and industrial users to develop faster and simpler nanoparticle detection and characterisation processes.

Potential future impact on industry

The reference nanomaterials and the measurement techniques developed by this project are the first of their kind, and will lead to a better understanding of the properties of current and future nanoparticles. They will play a key role in ensuring the safe use of nanomaterials; results will be used by nano-biotechnology and nano-medicine organisations to validate their protocols, and to perform toxicology studies and risk assessments. Regulatory bodies and legislators will benefit from a clearer understanding of the effects of nanomaterials on health, and will have a foundation upon which they can develop policies and guidelines. The important first step made by this project in the understanding of the properties of antibody-bound nanoparticles has laid the foundations for the increased use of nanoparticles in medicine and healthcare.

The achievements of this project have established avenues for additional scientific research, further developed in the EURAMET projects HLT02 MetVes (detection of microvesicles in body fluids) and 14IND12 Innanopart (measuring nanoparticle concentrations). The development of multi-method approaches for characterising nanoparticle properties has also complement the work of NanoDefine, a current EU project implementing a standardised EC definition of a nanomaterial. Ultimately, the results of this project will enable

European nanotechnology researchers and industry to develop new, safe, higher-performance products and processes.

List of publications

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C. Würth and U. Resch-Genger, *Determination of Photoluminescence Quantum Yields of Scattering Media with an Integrating Sphere: Direct and Indirect Illumination*, Applied Spectroscopy, Appl Spectrosc. 2015, 69 (6), 749-59, doi: 10.1366/14-07679.

A. Sikora, D Bartczak, D. Geißler, V. Kestens, G. Roebben, Y. Ramaye, Z. Varga, M. Palmai, A. G. Shard, H. Goenaga-Infante, and C. Minelli. *A systematic comparison of different techniques to determine the zeta potential of silica nanoparticles in biological medium*, Anal. Methods, 2015, 7, pp 9835-9843.

G. Roebben, et al., *Reference materials and representative test materials to develop nanoparticle characterisation methods: The NanoChop project case*. Frontiers in Chemistry, 2015, doi: 10.3389/fchem.2015.00056 (open access)

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