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NM-300 Silver
Characterisation, Stability, Homogeneity

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NM-300 Silver
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Abstract

The European Commission Joint Research Centre (JRC) provides scientific support to European Union policy regarding nanotechnology and public health in a sustainable environment. Over the last three years, the JRC has focused part of its work on establishing and applying a priority list (NM-Series) of Representative Manufactured Nanomaterials (RMNs) in support of one of the most comprehensive nanomaterial research programmes that is currently being carried out: the Organisation for Economic Co-operation and Development's (OECD) Working Party on Manufactured Nanomaterials (WPMN) Sponsorship Programme. The JRC's provision of NM-Series RMNs to the OECD WPMN Sponsorship Programme ultimately enables the development and collection of data on characterisation, measurement, toxicological and eco-toxicological testing, and risk assessment or safety evaluation of nanomaterials. Representative nanomaterials are of utmost importance to be made available to the international scientific community to enable innovation and development of safe materials and products.

The present report describes the characterisation of NM-300, a RMN nano-Silver dispersion containing nanoparticles (NPs) of < 20 nm, originating from a single batch of commercially manufactured nano-Silver, used for measurement and testing for hazard identification, risk and exposure assessment studies. The first series of sub-samples created from the batch is labelled NM-300, while the further processed series of NM-300 is labelled with an additional "K" as NM-300K, in order to signify a continued processed number of sub-samples from the same batch of raw material. The nano-Silver material was provided as a representative as-produced commercial nanomaterial and its NM-Series processed in the frame of the JRC programme on nanomaterials. It was studied by a number of international laboratories including the JRC IES analytical laboratory. Inorganic chemical characterisation of the total silver content and the homogeneity of the silver distribution were performed using photometry and ICP-OES. To this end, a dedicated method was developed and validated according to the principles of ISO 17025. Key properties of size and size distribution were studied in an inter-laboratory comparative study using SEM as

well as TEM and Nanoparticle Tracking Analysis. Furthermore, the release of silver ions from the NM-300 was studied after embedding in an acrylic matrix.

The NM-300 silver <20 nm was found to contain silver particles of about 15 nm size with a narrow size distribution of 99 % of the particle number concentration exhibiting a diameter of below 20 nm. A second, much smaller abundance of particles was identified by TEM to have narrow diameter distribution of around 5 nm. The silver content and particle number of NM-300 was shown to be stable over the time of examination, lasting up to 12 months. Silver-ions were released from NM-300 silver nanomaterial, which was embedded in a poly-acrylic matrix up to a defined concentration level.

The properties of NM-300 studied and described in this report demonstrate its relevance for use in measurement and testing studies, such as for hazard identification, related to the safety of nanomaterials. The studies were performed in close collaboration with the Fraunhofer Institute for Molecular and Applied Ecology (Fh-IME), German Institute of Energy and Environmental Technology e.V. (IUTA), the Swiss Federal Laboratories for Materials Science and Technology (EMPA), RAS Material Science GmbH, Germany, and the Veterinary and Agrochemical Research Centre (VAR) in Belgium.

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List of abbreviations

CEN	Comité Européen de Normalisation, Belgium
EMPA	Swiss Federal Laboratories for Materials Science and Technology, Switzerland
GDMF	General Decision Making Framework
GF-AAS	Graphite Furnace Atomic Absorption Spectrometry
GLP	Good Laboratory Practice
GUM	Guide to the Expression of Uncertainty in Measurement
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectrometry
Fh-IME	Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany
ISO	International Organisation for Standardization, Geneva, Switzerland
ISO/TC 229	ISO Technical Committee on Nanotechnologies
IES	Institute for Environmental and Sustainability (JRC), Italy
ITS	Integrated Testing Strategy
IUPAC	International Union of Pure and Applied Chemistry, Triangle Park, USA
IUTA	Institute of Energy and Environmental Technology e.V., Germany
JRC-IHCP	Institute for Health and Consumer Protection (JRC), Italy
LoD	Limit of Detection
LoQ	Limit of Quantification
NP	Nanoparticle
NM	Nanomaterial
NTA	Nanoparticle Tracking Analysis
OECD	Organisation for Economic Co-operation and Development, France
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
RM	Reference Material
RMN	Representative Manufactured Nanomaterial
RSD	Relative Standard Deviation
SD	Standard Deviation

SOP	Standard Operating Procedure
SCENIHR	Scientific Committee for Emerging and Newly Identified Health Risks
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
US-EPA	US Environmental Protection Agency
UV-VIS	Ultraviolet-visible Spectrophotometry
VAR	Veterinary and Agrochemical Research Centre, Belgium
WHO	World Health Organisation
WPMN	Working Party on Manufactured Nanomaterials
w/w%	weight percent
WWTP	Waste Water Treatment Plant

1 Introduction

Silver nanoparticles are most promising materials for a range of applications, due to the property of silver to be an antibacterial and antimicrobial agent (Morones *et al.*, 2005; Kim *et al.*, 2007). Comparable types of uses have been well known for a long time from medical applications and the field of biomedical devices. In biomedicine, vascular implants, such as coronary stents, catheters or orthopaedic devices have been designed using silver to better perform and function in their intended use and application (Laurin *et al.* 1987).

Different nano-Silver containing products were developed in these and other domains using its antibacterial activity, for example in recent applications as coating agent in textiles (Perelshtein *et al.*, 2008) or in wound dressing (Chen and Schluesener, 2008). According to the *Project on Emerging Nanotechnologies Inventory of nanotechnology-based Consumer Products* (Woodrow Wilson International Center for Scholars, 2009), silver nanotechnology is present in more than 240 commercial products, ranging from medical applications, domestic appliances and cleaning products, antibacterial textiles, food storage and personal care products and also some kids toys. These new nano-Silver-based products are nowadays part of everyday life, and hence in close contact with human beings and the environment. While nano-Silver containing products provide significant benefits due to their biocidal effects, little is conclusively described about their environmental fate, toxicity and eco-toxicity, respectively (Handy *et al.*, 2008).

In the last years, some toxicity studies were carried out on aquatic species (Asharani *et al.*, 2008), human cells (Greulich *et al.*, 2009) and mammalian cells (Ahamed *et al.*, 2008, Arora *et al.*, 2009). Moreover, in a recent article (Kvitek *et al.*, 2008) the attention was also posed on the possible increase of silver nanoparticles ecotoxical effects by the interaction with surfactants/polymers.

A possible emerging problem is the risk due to the release of silver nanoparticles (NPs) directly into wastewater, based on the increasing use of household products containing nano-Silver. In these products, the release of silver nanoparticles depends strongly on the method of fixation and embedding

into the respective matrix. In contrast to using nano-Silver as a polymer additive during the initial fibre-spinning process, the simple functionalization of textiles by coating can, in fact, result in fast release of silver during the time in their life cycle, like fabrics during regular washing (Benn and Westerhoff, 2008; Geranio et al., 2009), which is directly discharged into sanitary sewage system (Blaser et al., 2008; Benn and Westerhoff, 2008).

Figure 1 shows a schematic illustrating the potential release of silver into wastewater. (Blaser *et al.*, 2008): Wastewater from domestic sewer systems enters a wastewater treatment plant (WWTP) where most of the silver is removed and deposited in sewage sludge produced from waste treatment (Blaser *et al.*, 2008, Gottschalk *et al.*, 2009). Environmental contamination of silver can thus arise from the re-use of sludge, for example in agricultural soil fertilisation, giving rise to possible soil and groundwater pollution (Blaster *et al.*, 2008).
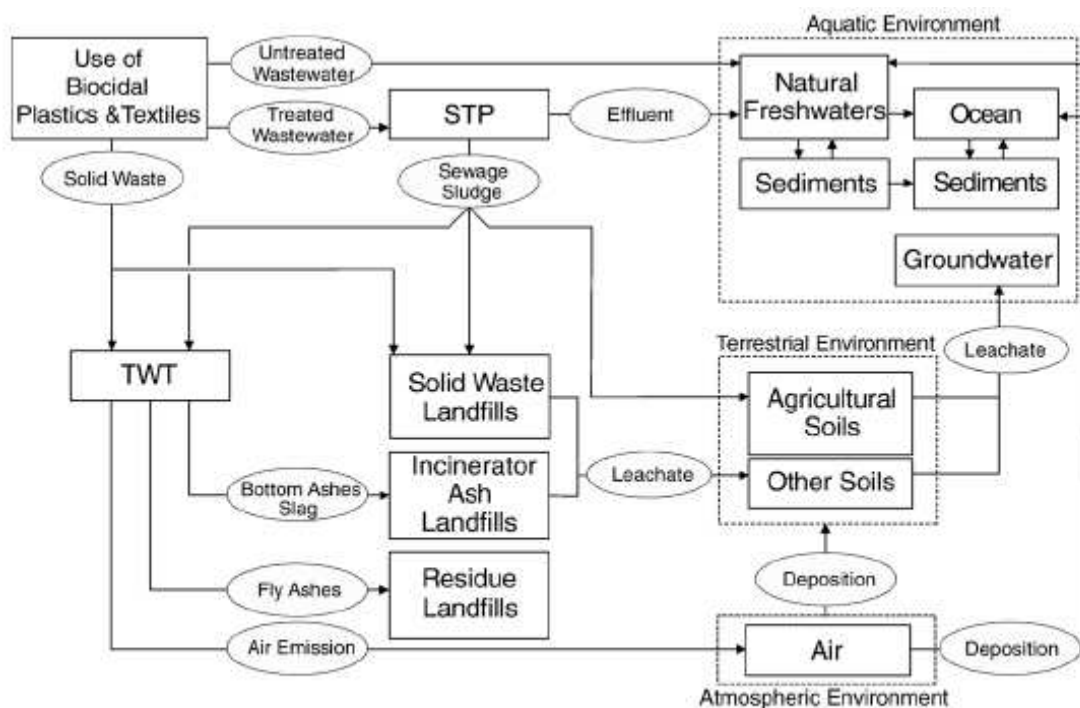

Figure 1: Silver flows due to silver containing products (by Blaser et al., 2008).

A modelling study concerning nanoparticle concentrations in the environment, conducted by Mueller and Nowack (Mueller and Nowack, 2008), revealed that the use of sludge as fertiliser releases about 1 $\mu g/kg^3$ nano-Silver per year,

based partly on the fact that 50% of agricultural land exclusively receives sludge from WWTP.

Considering the increasing use and developments of nano-Silver household products, the sewer system represents a major potential nano-Silver pathway. It is thus important, as a first approach, to correctly quantify the total silver content, both in sludge and effluents from WWTP, as it can independent of its form (*i.e.* nano or not) affect aquatic and terrestrial ecosystem. The total share of silver is also key to the development of reliable approaches, such as multivariate approaches, necessary for a large-scale assessment of nano-Silver environmental occurrence.

In order to reliably address the scientific questions of silver nanomaterial-induced effects, toxicity, ecotoxicity and fate, representative nanomaterials are required, which are relevant for industrial application and commercial use, and for which a critical mass of study results are generated or known. These Representative Manufactured Nanomaterials (RMN)s will allow comparison of testing results, the development of conclusive assessment of data, and pave the way for appropriate test method optimization, harmonisation and validation. They may serve as performance standards for testing.

The NM-300 (and NM-300K) nano-Silver < 20 nm was introduced by the European Commission Joint Research Centre to fulfil this function (with the "K" added to the NM-Series-code, signifying a new processed batch after the "first" NM-300 batch was exhausted, due to the huge interest by the scientific community for RMNs). The present report describes a number of NM-300 relevant properties and the applied methodology including size and size distribution by Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Nanoparticle Tracking Analysis (NTA). It addresses analyses of the silver content of NM-300 and its stability over a period of up to 12 months as well as homogeneity between vials.

From a methodological point of view, the results described in the report form a first analysis step for the development of a method aiming to quantitatively determine both the nano-Silver -related silver content in a sample and the total

silver content (i.e. including silver-ions in different forms, as well as other forms of silver) in the same sample. Such method could be transferred to other applications, such as determinations in sewage sludge and related effluents. The objective of these experiments was the validation of a method to measure silver in nano-form in wet samples, using ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) technology and microwave-assisted digestion, including the estimation of the measurement uncertainty.

2 Characterisation of representative nanomaterials

2.1 Representative Nanomaterials, reference nanomaterials, reference matrices and performance standards

Reference Material (RM) is the generic name for those materials which have a proven and sufficient homogeneity and stability in terms of a defined intended use. "Reference Substance" or "Reference Chemical" are terms used in toxicology for materials that need to meet similar conceptual requirements although being used for hazard identification usually under Good Laboratory Practice (GLP). RMs need to be produced and used applying the conditions and terms standardised and described in International Organisation for Standardization (ISO) Guides 30 to 35 for certification. When used in toxicology as test items, principles of Organisation for Economic Co-Operation & Development (OECD) Guidance Document (OECD GD) 34 and GLP apply *mutatis mutandis*. In addition, the IUPAC Group on Environment has introduced the concept of reference matrices, which describe representative and sufficiently complex materials that are used in testing approaches.

These criteria match as well the understanding of reference substances, representative materials, to be used as performance standards for test guideline test methods and regulatory use. The 'Sponsorship Programme for the Testing of Manufactured Nanomaterials' of the OECD Working Party on Manufactured Nanomaterials (WPMN) uses the term "Representative Nanomaterial", and the analogue term "Reference Nanomaterial"; these are represented in the NM-Series of Representative Manufactured Nanomaterials (RMNs).

2.2 Use of NM-Series RMNs

RMs can be used for different purposes, such as calibration, assessment of laboratory proficiency or test method performance. Only a small number of reference materials already exist in the field of manufactured nanomaterials (*e.g.* gold nanoparticles from the National Institute of Standards and Technology, USA and silica from the European Commission, Joint Research Centre, Belgium), but they are spherical model materials which are certified primarily for size and are used mainly to calibrate instruments, which measure

particle size. The absence of well-defined measurands and standardised test protocols is identified as a major obstacle for RM production, because agreed and harmonised methods are needed.

A typical issue of studies performed, such as under the Sponsorship Programme of the WPMN, is to link a strong metrological part under ISO 17025 principles with dedicated knowledge about reference material production under ISO Guide 30 to 35 with an intended use in toxicological test systems under GLP complemented by information about dosage and routes of exposure. In addition, regulatory requirements could play a role, such as the need for GLP compliance, when used in the framework of the General Decision Making Framework (GDMF) of the European REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) regulation, following Article 13 of Regulation EC 1907/2006 and Directive 2004/10/EC. Correspondingly, the ISO standards refer to "materials", whereas European legislation, REACH and GLP addresses "substances". 'Shopping lists' for desired property information have been established or are under development, such as by OECD WPMN and ISO/TC 229 WG3 (Working Group 3: Health, Safety and Environmental Aspects of Nanotechnologies). Current documents unfortunately do not give guidance which parameters to measure, which methods to use, under which conditions, when and at which point of the process or even for which purpose. This point was made in many original publications, and distinctions are made between intrinsic and extrinsic properties by SCENIHR and International Union of Pure and Applied Chemistry (IUPAC) on how data acquired can be used in the process of risk assessment. The IUPAC Group on Environment has published the concept of "Reference Matrices", which describe representative, complex materials, which are used in testing approaches. The group describes the need to develop reference matrices, which are sufficiently near to real life substances/materials to be used for testing and risk assessment in corresponding test systems. This nicely matches the understanding of reference substances used as performance standards, such as described under OECD GD34, whereas dedicated materials that have a narrow scope of variation of a particular property, such as size, are useful for mechanistic studies during the

development and optimisation phase of a test system or for calibration of a measurement system. Nevertheless, it is likely that such materials can only be used in a particular test and even a particular Standard Operating Procedure (SOP) as control material.

In addition, current approaches do not address preparation of test items, which are suitable for being used in the respective test systems according to the technical, scientific and legally binding conditions. This is identified as a critical issue to be addressed with priority as it provides the basis for appropriate testing in the Integrated Testing Strategy (ITS) and appropriate modelling *in silico*. Although deeper discussions on terminology and definitions are ongoing, questions of best practice today, its application and identified major pitfalls remain unsolved or even not addressed in this interdisciplinary context of physics, material science, measurement science metrology, process engineering, surface and applied chemistry, pathobiology, and toxicology.

2.3 Scenarios for characterisation of NM-Series RMNs

The OECD WPMN in its work in Steering Group 7 (SG7) on The Role of Alternative Test Methods in Nanotoxicology focuses to address the associated practical issues in close cooperation with the sponsorship programme under SG3 (Steering Group 3 on Safety Testing of a Representative Set of Manufactured Nanomaterials) and SG4 (Steering Group 4 on Manufactured Nanomaterials and Test Guideline) in an interdisciplinary approach.

In practice, and in agreement with the requirements mentioned above, characterisation results should be gained and used in their appropriate context scenarios.

Scenario 1 (i.e. "as in the Repository") is the characterisation of the intrinsic properties of a representative (reference) nanomaterial (NM). A number of intrinsic properties need to be determined for each NM. The physical state and preparation form of the material examined should thereby be representative for production and use, taking into account the chain of actors and life cycle. Typical preparation forms are dry or aqueous. Sample preparation steps corresponding to analytical sample preparation should be critically assessed

with regard to being a determinant of the measurement result itself, such as particle size determinations dry, in aqueous solution or physiological media. A careful selection of parameters is used to assess stability and homogeneity of the reference nanomaterials in the repository of NMs hosted at the European Commission JRC.

Matrix-Sample conditioning and processing:

Test item preparation: Before entering the next stage, protocols are developed for test item preparation for use in test systems for toxicological evaluation or environmental fate analysis. This comprises conditioning and choice of matrix components. The prepared test item should thereby correspond to the requirements of the test method and GLP, and typically be representative for the identified exposure taking into account the chain of actors and life cycle. Test items are prepared for oral, dermal, (intravenous) and inhalation toxicity testing for human health and for application to environmental compartments soil, water and air in the form, which is envisaged to reach the biological entity in the test system. Representative nanomaterials can best be used and brought into a matrix under defined conditions, while applying defined procedures. The preparation protocols for test item and definition of corresponding matrices will guarantee a state of the art standardisation and harmonisation of protocols, which were identified as a major source of uncertainties or methodological errors.

Scenario 2 (i.e. "as prepared test item") comprises the characterisation of matrix-dependent properties following the steps of test item preparation and corresponding to the prepared test item. Some authors use the term "extrinsic" properties for this scenario. Results are dependent on the matrix components and protocols used. Several results may be gained for the same measurement parameter, depending on the applied conditioning protocol and chosen matrix, as it is well established that particle toxicity is not only determined by a particle's chemical composition. The effect of a particle's 'corona', the molecules surrounding it in a given medium, has recently been acknowledged, emphasising that the corona is sensitive to the medium.

Biophysical characterisation, such as corona composition, kinetics/ exchange rates, corona structure and depletion effects/changes in matrix kinetics is therefore required in support of understanding of test items.

Indeed, shape, size and surface area affect the hazards associated with nanomaterials, due to a number of reasons, including the basic principle by which these parameters affect the transport properties of the particles (absorption, distribution, and excretion). Representative (Reference) nanomaterials have to be seen in their context of intended use. They are tools of Quality Assurance and method validation, they serve method harmonisation and standardisation and performance assessment.

The European Commission JRC has developed representative NMs in accordance with the ISO Guide 30 to 35 principles. The well characterised NMs are being used in the toxicology- and fate-studies following the principles of the guidance document on the validation of test methods for hazard assessment, OECD GD 34 and the international test method validation principles.

The understanding of the principle of these scenarios and how the test item or sample preparation steps may change and influence the determined value for a selected property of an NM, is of crucial importance to make best use of tools of computational toxicology and to gain an understanding of the information content and use of data acquired, and at which point more data is required to make reliable predictions, and support grouping, categorisation approaches and read-across.
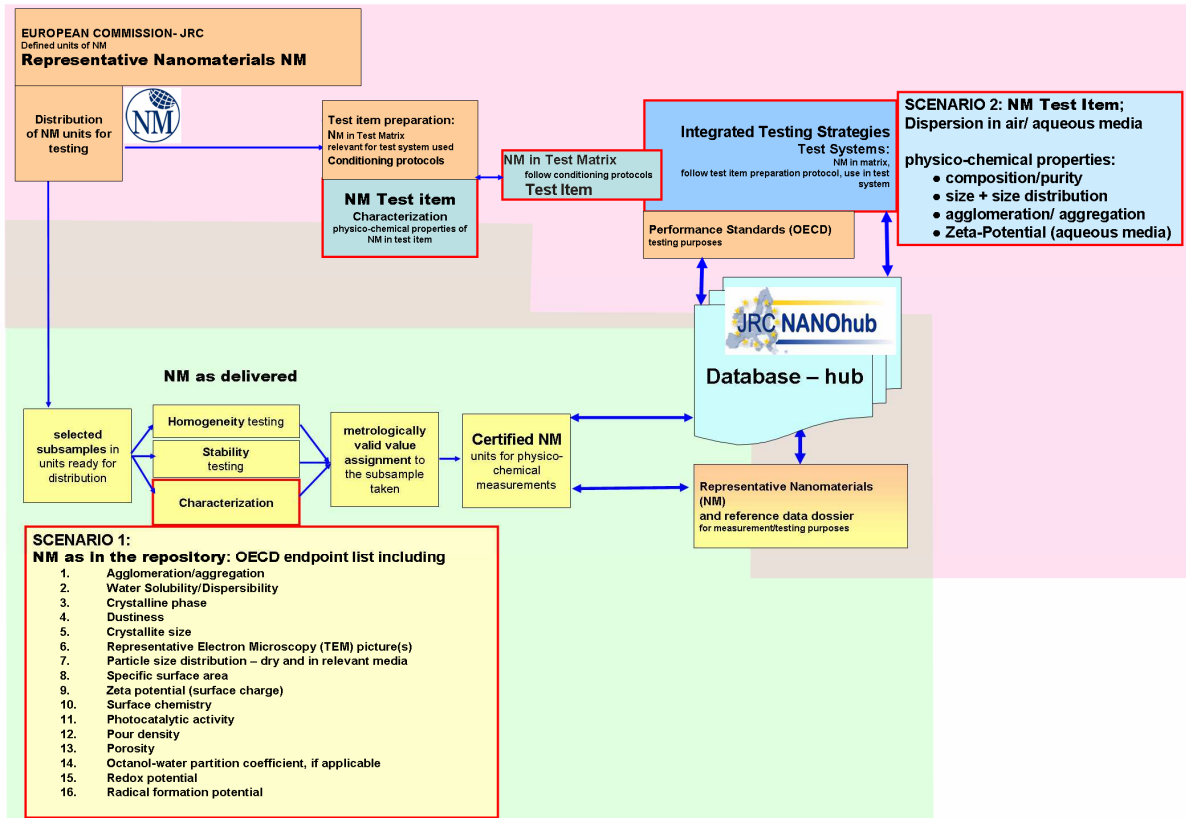
Figure 2: NM characterisation and properties under scenario 1 "as in the Repository" and scenario 2 "as prepared test item".

3 JRC Repository of NM-Series of Representative Manufactured Nanomaterials and the OECD Sponsorship Programme

Nanotechnology holds considerable promise in many technological areas and industrial sector and the application of nanosciences and nano-structured materials to everyday products offers a range of benefits; their application in every-day consumer products may make the latter lighter, stronger, cleaner, less expensive, more efficient, more precise, more functional, more durable, and also more aesthetic. Products with specific properties derived from nanotechnology currently available on the market include textiles, cosmetics and beauty products, water filters, food, food-packaging materials, paints, glues and dental fillers. Nanomaterials may also improve our quality of life via their use in applications leading to more efficacious pharmaceuticals, improved medical diagnostic tools and faster computers, to name but a few. This has been matched by growth in requests for characterised representative nanomaterials for use as reference matrices for testing to reliably address health and safety concerns for humans and the environment related to nanomaterials and corresponding implementation of European policy and responsible nanotechnology decisions (Morris *et al.* 2011).

3.1 JRC Repository of NM-Series of RMNs

The European Commission Joint Research Centre acts as independent agent connecting science and regulators, Member States, industry, non-governmental organisations and international stakeholders and organisations. This comprises a direct link to harmonization and standardization at the Organisation for Economic Cooperation and Development (OECD) and its Working Parties, the Comité Européen de Normalisation (CEN) and the International Organisation for Standardization (ISO). Fulfilling its unique roles, the JRC has established a repository for the NM-Series of RMNs, which today hosts more than 25 different types of NM-Series of RMNs and a total number of more than 20000 individually labelled samples at its centre in Ispra, Italy. JRC-IHCP has introduced the NM-

Series of RMNs for the measurement and testing in support to the Organisation of Economical Cooperation and Development (OECD) - Working Party on Manufactured Nanomaterials (WPMN) as well as European and Member States' projects. Several NM-Series are already being studied in international scientific co-operations. More than 10000 individual test samples were distributed internationally to National Authorities, research institutions, industrial research laboratories and other scientific stakeholders in the EU, Switzerland, USA, Canada, Australia, China, Russia, Japan, and Korea. Study results are collated in a JRC database and are made available to the OECD through dedicated data submissions to the JRC NANOhub database, which is hosted at JRC.

Together, representative nanomaterials and reference data, such as from the JRC NANOhub are enabling innovation and competitiveness in Europe's growing nanotechnology industries by building foundations for research and product development, such as for new and improved products and initiation of public-private partnerships to generate and analyse safety of materials and products.

3.2 NM-Series use at the OECD

The Organisation for Economic Co-operation and Development's (OECD) Working Party on Manufactured Nanomaterials (WPMN) is carrying out one of the most comprehensive nanomaterial research programmes: the OECD WPMN Sponsorship Programme for the Testing of Manufactured Nanomaterials. At JRC-IHCP, our primary goal is to provide scientific support to European Union policy regarding nanotechnology and public health in a sustainable environment. Within the WPMN, the JRC's work has been focused on a priority list of Representative Manufactured Nanomaterials (RMNs), for which development of data would support characterisation, measurement, toxicological and eco-toxicological testing, and risk assessment or safety evaluation of manufactured nanomaterials. The endpoints addressed within the OECD Sponsorship Programme are presented in Table 1.

Table 1: Endpoints addressed for the NM-Series of Representative Manufactured Nanomaterials.

OECD endpoints according to the Guidance Manual for Sponsors (GMS)	GMS- data requirements
NM name	must be completed
CAS No	if available
structural formula/molecular structure	must be provided
composition of NM being tested including purity, known impurities or additives	must be provided
basic morphology	must be provided
description of surface chemistry	if feasible
major commercial uses	as completely as possible
known catalytic activity	should be described
method of production	must be described
Identification, source, logistics of distribution (Guidance manual for Sponsors §41 and 42)	
known aspects: manufacturer, facility location, lot number, other, see above	must be completed
records on distribution, shipment, storage	must be completed
quality of material: homogeneity within bottle/ between bottles	must be completed
quality of material: stability, short-term and long-term	must be completed
quality of material: stability, monitoring	must be completed
Physical-chemical Properties and Material Characterization	
Agglomeration/aggregation	must be addressed
Water Solubility/Dispersibility	must be completed
Crystalline phase	must be completed
Dustiness	must be addressed
Crystallite size	must be addressed
Representative Electron Microscopy (TEM) picture(s)	must be addressed
Particle size distribution – dry and in relevant media	must be completed
Specific surface area	must be completed
Zeta potential (surface charge)	must be completed
Surface chemistry	must be completed
Photocatalytic activity	must be addressed
Pour density	must be addressed
Porosity	must be addressed
Octanol-water partition coefficient	must be addressed
Redox potential	must be addressed
Radical formation potential	must be addressed
Other relevant Physical-Chemical Properties and Material Characterization information (where available)	must be addressed
Environmental Fate	
Dispersion stability in water	must be addressed
Biotic degradability	must be addressed
Identification of degradation product(s)	must be addressed
Further testing of degradation product(s) as required	must be addressed
Abiotic Degradability and Fate	must be addressed
Adsorption-Desorption	must be addressed
Adsorption to soil or sediment	must be addressed
Bioaccumulation potential	must be addressed
Other relevant environmental fate information (when available)	must be addressed
Environmental Toxicology	
Effects on pelagic species (short term/long term)	
Effects on sediment species (short term/long term)	
Effects on soil species (short term/long term)	must be addressed
Effects on terrestrial species	must be addressed
Effects on microorganisms	must be addressed
Effects on activated sludge at WWTP	
Other relevant information (when available)	if available
Mammalian Toxicology	
Pharmacokinetics/Toxicokinetics (ADME)	must be addressed
Acute toxicity	must be addressed
Repeated dose toxicity	must be addressed
Chronic toxicity	must describe any relevant exsting study
Reproductive toxicity	must describe relevant reproductive toxicity test results
Developmental toxicity	
Genetic toxicity	must describe any relevant existing genetic toxicity test results
Experience with human exposure	must describe any relevant experience with human exposure
Other relevant test data	should be considered
Material Safety	
Flammability	if available
Explosivity	if available
Incompatibility	if available

The range of RMNs used within the OECD WPMN Sponsorship Programme are hosted in the NM Repository at the European Commission JRC in Ispra, where the RMNs are sub-sampled in collaboration with the German Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) under Good Laboratory Practice (GLP) conditions.

The NM-Series of RMNs currently includes: Carbon nanotubes, silver nanoparticles, titanium dioxide, cerium oxide, zinc oxide, bentonite, gold and silicon dioxide. These representative nanomaterials provide researchers with characterised materials, for which a globally agreed number and quality of studies are performed for potentially regulatory relevant endpoints. A selection of NM-Series RMNs kept at the JRC NM Repository is listed in Table 2. The NM-Series RMNs may be used by scientists in their testing models, and utilised as performance standards and comparators.

Table 2: List of selected NM representative Nanomaterials in the JRC NM Repository.

NM-Code	Label code	
NM-100	Titanium dioxide	anatase, pigment
NM-101	Titanium dioxide	anatase
NM-102	Titanium dioxide	anatase
NM-103	Titanium Dioxide	thermal hydrophobic
NM-104	Titanium Dioxide	thermal hydrophilic
NM-105	Titanium dioxide	rutile-anatase
NM-110	Zinc oxide	uncoated
NM-111	Zinc oxide	coated Triethoxycaprylylsilane
NM-200	Synthetic Amorphous Silica	PR-A-02
NM-201	Synthetic Amorphous Silica	PR-B-01
NM-202	Synthetic Amorphous Silica	PY-AB-03
NM-203	Synthetic Amorphous Silica	PY-A-04
NM-204	Synthetic Amorphous Silica	PR-A-05
NM-211	Cerium (IV) Oxide	
NM-212	Cerium (IV) Oxide	
NM-300	Silver, < 20 nm	
NM-300DIS	Silver dispersant	matrix control
NM-300K	Silver, < 20 nm	
NM300K DIS	Silver dispersant	matrix control
NM-400	Multi-Walled Carbon Nanotubes	
NM-401	Multi-Walled Carbon Nanotubes	
NM-402	Multi-Walled Carbon Nanotubes	
NM-403	Multi-Walled Carbon Nanotubes	
NM-600	Bentonite	

4 Materials and Methods

The first part of this section describes the equipment used both for the NM-300 size characterisation (performed by IUTA, EMPA and VAR) and for the ICP-OES analysis. The second part describes the methodology employed for mineralization and ICP analysis of NM-300 material.

4.1 NM-300 Representative Manufactured Nanomaterial

The experiments were conducted using NM-300 nano-Silver < 20 nm reference nanomaterial, which is used in a variety of studies and projects including the OECD WPMN Sponsorship Programme. The material is a nano-Silver colloidal dispersion with a nominal silver content of 10 w/w%. The NM-300 sample dispersion is yellow-brown; it is an aqueous dispersion of nano-Silver with stabilizing agents, consisting of 4% w/w% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20). The sub-sampled NM-300 was distributed by the Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg (Germany). Upon receipt at the Joint Research Centre in Ispra (Italy), samples were stored in the dark at 4 ºC.

The first lot of NM-300, processed under Good Laboratory Practice, proved so popular in the international testing and measurement community, that sub-samples were rapidly exhausted, so that a second sub-sample processing run was performed analogous to the first, using the same initial material batch for processing and exactly the same standard operating procedure (SOP) under GLP. According to labelling convention and analogous to the convention used for certified reference materials, the NM was attributed with the label name NM-300K. Findings reported are therefore analogously applicable to NM-300K.

4.1.1 Handling Procedure for Weighing and Sample Introduction

A handling procedure has been established in cooperation with scientists at the different research institutions, which used the NM-300 and NM-300K, respectively. It takes into account that the material is a highly concentrated nano-Silver dispersion. The nanoparticles have the tendency to sediment slowly and should be homogenised within the vial before use by vigorously shaking the sample. Artefacts have been observed in a few cases consisting of larger

aggregates or particles. In some cases, such aggregates were observed, when the content of the NM vial was not discarded, but re-used a long time after opening. The NM vial contains an Argon atmosphere. If the vial is not kept upright, or under the correct conditions, or if remaining dispersion is drying at the edge of the vial, artefacts, such as larger aggregates may form. NM vials, which were suspected to show such artefacts, have been replaced. Dedicated sample and test item preparation protocols need to be used depending on the specific requirements of the measurement procedure or the test method, such as outlined below regarding ICP-OES.

The suggested handling protocol for NM-300 reads:

BE FAST, once the vial is open! If possible, work in a glove box under inert dry atmosphere.

The vial containing the NM material is filled with Argon. Keep the vial upright. Record the individual sample ID number as indicated on the NM label. If working outside the glove box, wear latex gloves (or equivalent).

1)   record laboratory conditions including relative humidity of the laboratory air for QA,

2)   weigh a volumetric flask without cap,

3)   shake the vial before use: Make sure the vial is closed. Shake the vial vigorously for four minutes.

4)   remove cap from the NM-300K material vial,

5)   transfer an amount of dispersion into the volumetric flask using a pipette, determine and note down the weight of the volumetric flask with the transferred amount of NM-300K material,

6)   close the NM-300K material vial,

7)   calculate mass difference, corresponding to the weight of transferred amount of NM-300K,

8)   create desired dispersion by adding Ultrapure (Type I) water quality as described in US-EPA, EP and WHO norms,

9)   close the volumetric flask.

10   use this master stock dispersion for testing, accordingly.

General remarks:

A new pipette tip has to be used for each measurement.

Use Ultrapure (Type I) water quality as described in US-EPA, EP and WHO norms for dilution.

Store diluted samples in a refrigerator at 4 ºC in the dark, but keep time before use to a minimum.

4.2 Transmission Electron Microscopy (TEM)

The TEM experiments were performed at three independent institutes and three different TEM instruments and analysis tools were used.

The microscope employed by IUTA was a Philips CM12 with an accelerating voltage of 120 kV. The maximum deviation of the image analysis results based on the TEM images captured was 2%. The particle suspension (about 1 µL) was applied directly to the copper grids of the TEM, which were coated with a 20 nm thick layer of amorphous carbon. The microscope employed by VAR was a Tecnai™ Spirit (FEI) with an accelerating voltage of 120 kV. Digital micrographs were made using the bottom-mounted 4x4 K Eagle Camera using the TIA software.

The microscope employed by EMPA was a Zeiss 900 (Zeiss SMT, Oberkochen, Germany) with a 80 kV accelerating voltage and a CCD camera (Trödle, Moorenweis, Germany).

4.3 Scanning Electron Microscopy (SEM)

The SEM instrument employed by IUTA, was a LEO (Zeiss) 1530 with an accelerating voltage of 10 kV. It was calibrated by means of a Chessy sample before starting the measurement. The particle suspension (about 5 µl) was applied directly to the monocrystalline silicon substrate.

4.4 Nanoparticle Tracking Analysis (NTA), Dynamic Light Scattering and Zeta-Sizer Analysis

The NT Analyser, employed by EMPA, was a NanoSight LM 20 System (NanoSight Ltd., Amesbury, Wiltshire, UK). Dynamic Light Scattering experiments and zeta-sizer analysis were performed by a number of scientists using a variety of instruments within the frame of collaborative projects, *e.g.* funded by the European Commission. The collaborative projects were using the NM-300/NM-300K.

4.5 Stability of NM-300 b UV-VIS Spectrophotometry (UV-VIS)

Spectrophotometrical analysis of the NM-300 nano-Silver dispersion was performed by using a Hitachi U-2000. The sample was prepared by diluting 100 mg of NM-300 in 500 g water.

UV-spectra are generated using a 1 cm cuvette for wavelengths 700 nm to 350 nm. The absorbance maximum and the half width of the peak are determined. The amount of nano-Silver content in weight-% is determined by using the basic correlation according to the established correlation calibration plot established by comparison of UV-VIS with GF-AAS determination results.

4.6 Silver-Ion Release analysed by GF-AAS: Different NM-300 Content and Elution Media, forced Release and Long-Term Studies

A number of experimental studies were performed regarding the content of silver-ions in the NM-300 representative nanomaterial, which was embedded in a poly-acrylic matrix and the subsequent elution of silver ions under varying conditions. NM-300 nanomaterial was embedded into strips of poly-acrylic, with each strip having a mass of 580 mg and a surface of 460 mm^2. After impregnation with NM-300, the poly-acrylic strips were rinsed with double-distilled water and stored under controlled elevated temperature (*i.e.* 37 °C) in the following media to determine the equilibrium constant:

- physiological phosphate buffer,
- 0.5 % nitric acid,
- de-ionized water, and
- the NM-300 matrix.

The release of silver-ions into the elution matrix was measured for up to 13 days without removing and exchanging the elution media. The release of silver-ions into the media was studied as a function of NM-300 concentration embedded into the poly-acrylic matrix, amounting to silver-concentrations of 42 µg/g up to 4000 µg/g. Furthermore, the influence of temperature on the release of silver-ions was investigated by heating the samples to up to 100 °C. In addition, the release of silver from poly-acrylic strips doted with 2500 µg/g was examined for a period of up to 33 days under ambient temperature, for which the elution media were removed and replaced with fresh medium after each period of 5 days.

An incubator (Koettermann shaker-incubator water-bath) was used to store the leaching samples under a constant controlled temperature; acrylic sample strips were covered with 1.0 ml elution media in a 1.5 ml vial. Three parallel samples were examined for each treatment group. Control groups were studied, which contained no NM-300 nano-Silver. Three samples each were retrieved at the respective time for analysis by Graphite Furnace Atomic Absorption Spectrometry (GF-AAS). Determinations by GF-AAS were done in triplicate.[1]

A Solaar Unicam 989 AA spectrometer was used conduct the GF-AAS measurements. The graphite oven consisted of Solaar Unicam GF 98 plus furnace with sampler FS 90 and a furnace auto-sampler. Coated graphite sampling tubes were used as well as a deuterium lamp and silver-cathode lamp with a resonance wavelength for silver of $\lambda = 328.1$ nm; the spectral gate applied was 0.5 nm.

4.7 Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES)

Silver analysis was carried out with the Optima 2100 DV ICP-OES (Inductively Coupled Plasma with Optical Emission Spectrometry) by Perkin Elmer. The used conditions are listed in Table 3.

Table 3: Operational conditions of the ICP-OES.

Parameter	
Plasma condition	
Plasma flow (Argon)	15 l/min
Auxiliary flow (Argon)	0.2 l/min
Nebulizer flow (Argon)	0.8 l/min
Power	1300 W
View distance	15.0
Plasma view	Axial
Peristaltic pump	
Sample flow rate	1.5 l/min
Autosampler	
Wash between samples for 30 s	

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[1] Graphite furnace atomic absorption spectrometry (GF-AAS) is also known as Electrothermal Atomic Absorption Spectrometry (ETAAS), is a type of spectrometry that uses a graphite-coated furnace to vaporize the sample. During the heating process, free atoms thereby absorb light at frequencies or wavelengths characteristic of the element of interest.

Silver was measured at a wavelength of 328.069 nm and the under-peak area was used for further spectral peak processing.

4.7.1 Microwave System

Microwave digestion of the samples was conducted utilising a Milestone Ethos 1600 microwave, with an installed maximum power of 1600 W. Analyses can be performed on up to ten samples simultaneously. Microwave digestion conditions employed for NM-300 are listed in Table 4 and reagents are given in 4.7.3 below.

Table 4: Program used for microwave digestion (Vent.=ventilation).

Time	Power
7	250
7	500
5	750
3	Vent.

The programme listed in Table 4 was previously optimized for sludge analysis.

4.7.2 Balance

The electronic balance used to weight employed materials was a Mettler AT261 (Mettler Instruments Corp., Hightsown, NJ) with precision of 1 µg.

4.7.3 Reagents

The following units of NM-300 were received: *0021, 0030, 0033, 0051, 0075, 0886, 0897, 1048, 1170, 1432, 1468, 1493, 5061, 5062, 5063, 5064, 5065, 5066, 5067, 5068*. Trace analytical-grade nitric acid (HNO_3) 65% and hydrochloric acid (HCl) 37% were used in microwave digestion. Milli-Q water (18.2 MΩ cm) was used for dilutions.

Silver ICP stock solution 1000 µg/ml, Ultra Scientific ICP-047, diluted in nitric acid 2%, Lot Number E00317 was used as ICP standard.

4.7.4 Dilution

The NM-300 silver dispersion has a nominal concentration of 10 w/w% and appropriate dilution procedure was applied, following the sample preparation protocol described above (see: 4.1 NM-300 Representative Manufactured Nanomaterial) to obtain a concentration more suitable for ICP analysis.

As the material is very viscous and some solution could remain in the pipette tip during the collection of an aliquot, a gravimetric approach was used to obtain the solutions prior to analyses. This procedure was also recommended as input in the general handling procedures of materials similar to NM-300.

For dilution, approximately 25 µl of NM-300 silver solution were transferred into a 100 ml pre-weighted glass flask using a pipette. The exact sample intake was then determined with the electronic balance. Upon weighing, the flask was filled to volume with analytical-grade Milli-Q water and shaken to homogenise the content.

In order to determine the approximate silver concentration in mg/kg in the diluted solution prior to the respective analytical step of determination using ICP-OES, the density of the NM-300 nano-Silver dispersion was computed.

4.7.5 Density Computation

For density computation, the NM-300 sample with identification number "**0060**" was used. After gentle shaking of the vial for about four minutes, a known amount of solution (50 µl) was collected using a pipette and weighed. The procedure was repeated ten times in order to average the measured density value, with the average density being the ratio between the average weight and the known volume.

Table 5 shows the density calculation results. *Volume* is the amount of NM-300 silver solution weighed.

Table 5: Density calculation results.

Measure	Volume (µl)	Weigh (g)
1	50	0.0550
2	50	0.0551
3	50	0.0508
4	50	0.0553
5	50	0.0568
6	50	0.0550
7	50	0.0552
8	50	0.0557
9	50	0.0560
10	50	0.0548

The average density is equal to 1.10 kg/l with standard deviation of 0.03 kg/l.

4.7.6 Microwave Digestion

For the microwave-assisted digestion, 200 µl of diluted NM-300 solution were transferred into the digestion vessel under a fume hood. Subsequently, 3 ml of HCl and 1 ml of HNO_3 (*i.e.* a defined mixture known as *'aqua regia'*) were added. A blank (3 ml of HCl and 1 ml of HNO_3) was added to each digestion series.

The type and amount of reagents used in the digestion reflect those of a previous study based on the optimization of mineralization procedure for sludge. Vessels were closed put into the microwave oven; and the digestion programme described in Subsection 4.7.1 was started.

At the end of the microwave programme, digestion vessels were left to cool to ambient temperature and then they were opened under a fume hood.

Each extract was filtered in a 50 ml glass flask using a clean glass funnel and a Whatman filter (grade No. 42). The vessel and the vessel cup were subsequently rinsed three times with Milli-Q water and the rinse water was filtered in the same flask. At the end, the flask was completed to volume. The resulting samples were stored at 4 ºC until analyses.

4.7.7 ICP-OES analysis

For ICP analysis an aliquot of the digested samples was transferred to the ICP sample holder vials. For the calibration curve, a standard blank and an appropriately diluted amount of the silver standard solution were used (see next Section: 4.8 Method Validation).

4.8 Method Validation for quantitative Silver Determination by ICP-OES

The method validation for the analysis of total silver content of the NM-300 RMN using ICP-OES technology and microwave assisted digestion, was conducted referring to ISO 17025 (ISO/IEC 17025: 1999). NM-300 vial with sample identification number 0060 was used.

4.8.1 Calibration Study

In order to verify the linearity of the calibration curve, one blank and five standard concentrations were analysed in three replicates for five different days. Standards were obtained by diluting the silver stock solution in 100 ml flasks with Milli-Q water and 2 ml of HNO_3. The five standard concentrations used for calibration had a concentration of: 0.03 mg/l, 0.05 mg/l, 0.1 mg/l, 0.3mg/l and 0.5 mg/l.

Linear calibration curves for each daily calibration are shown in Figure 2 and respective correlation coefficients are reported in Table 6 only for the calibration conducted on the first day the Regression coefficient (R-value) is smaller than 0.9999.

Table 6: Regression coefficients of linear calibration curves.

	Day 1	*Day2*	*Day3*	*Day4*	*Day5*
Regression coefficient	0.9992	> 0.9999	> 0.9999	> 0.9999	> 0.9999

Figure 3 shows that the shape of the calibration curves and the regression coefficient values for each daily calibration prove the linearity and stability of the measurement system (silver diluted standards and ICP instrument) in the 5-days range; calibration curves were also obtained after experiment durations of up to 9 days, confirming the stability of the system.
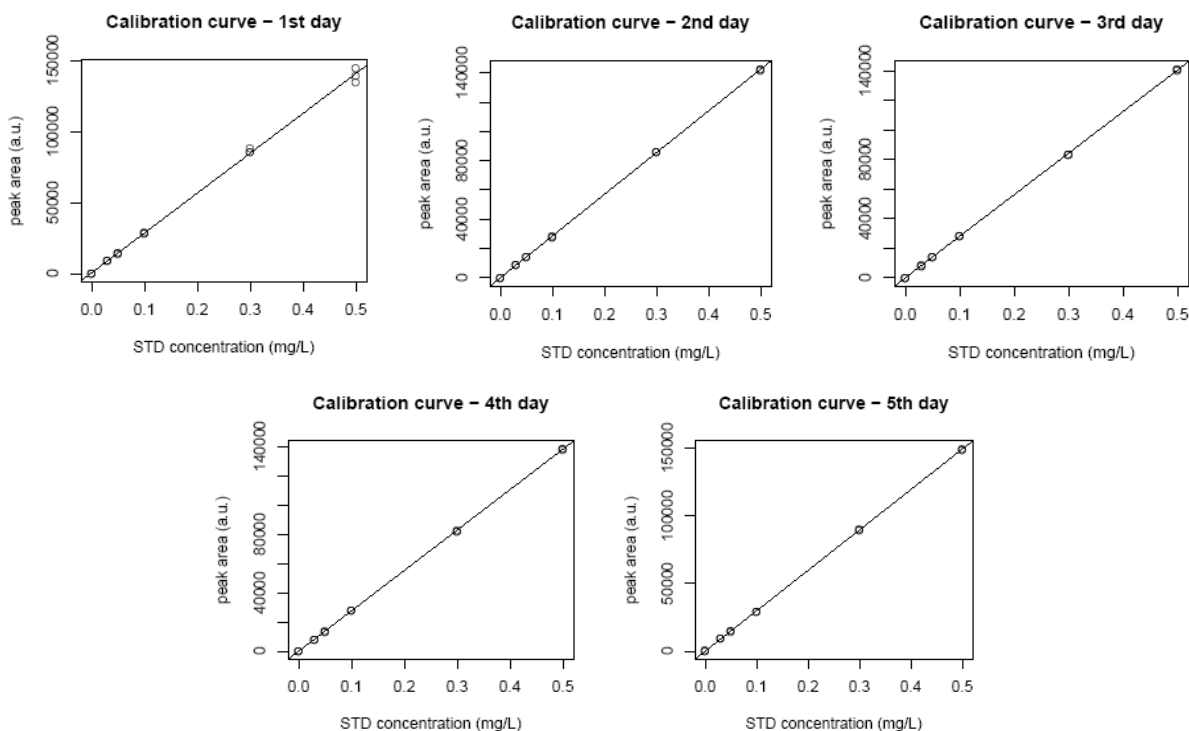
Figure 3: Linear calibration curves for silver.

However, as correlation coefficients are not the best parameter to prove the linearity (Loco *et al.*, 2002; González and Herrador, 2007), another method was applied to additionally check the linearity of the plots and thus ultimately verify the stability of NM-300: residual plots, with residuals being the difference between the computed y-value and the predicted one using calibration function, were performed.[2] In Figure 4, plots of standardized residuals are reported to prove the linearity. No trend is observed for each daily calibration, confirming linearity.

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2 Residual plot are used to graphically show deviation from the chosen curve and data homoscedasticity (Thompson et al., 2002).
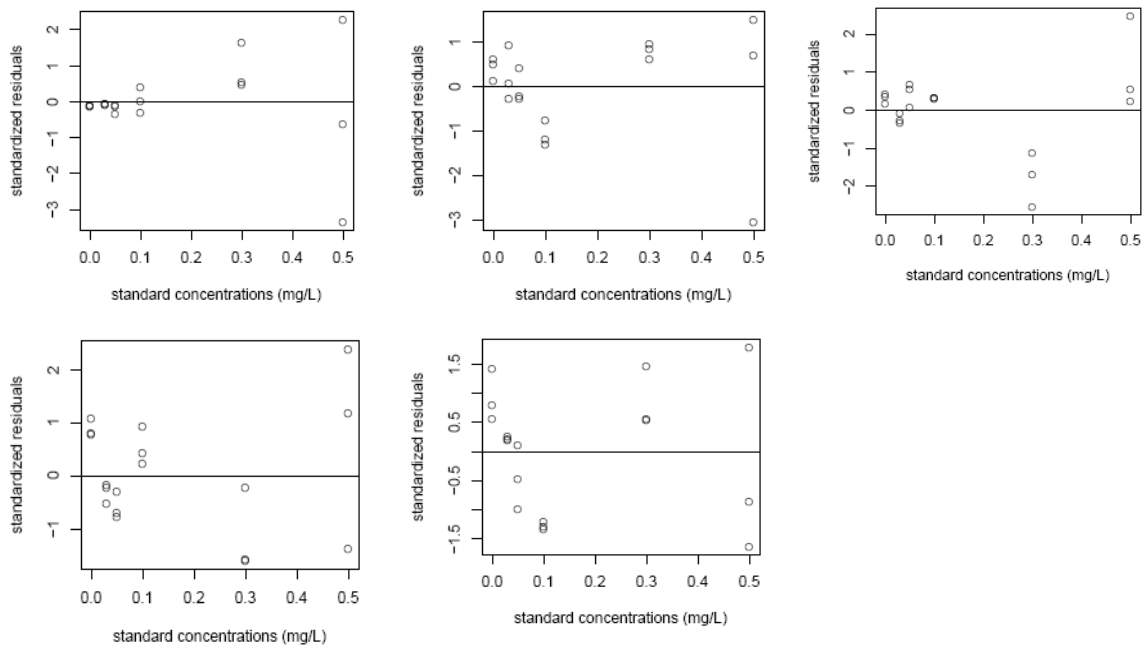
Figure 4: Residual plot for low calibration.

An F-test was used to confirm the linear trend of the regression curve expressed by the correlation coefficient.[3] The F-test shows that the calibration model is suitable for all daily calibration curves, at the 99% confidence level; for the first two days, the linear model adequately fit the calibration data at the 95% confidence level.

In order to test the homogeneity of variance, Bartlett's test and Fligner-Killeen's test were applied. The first one is more sensitive to non-normality of data, while the Fligner-Killeen's test is more robust in the case of departure from normality.

Bartlett test reject the hypothesis of homogeneity of variance for all calibration curves while Fligner-Killeen's is significance for all calibration curves at the 95% confidence level.

4.8.2 Working Range

The working range is defined by the calibration curve (upper value) and the Limit of Quantification (LOQ, see Subsection 4.8.3 below). For higher concentration than those defined by calibration curve the measured solution has to be diluted and re-analysed.

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3 The 'lack of fit'-test helps to determine if the modelling error is significant different than the pure error and this is done comparing the variance of the lack of fit against the pure error variance (González and Herrador, 2007.).

Limit of Detection / Limit of Quantification

In order to estimate LoD (Limit of Detection) and LoQ (Limit of Quantification), a sample containing the analyte under analysis at very low concentration was analysed. Ten replicates were made, in order to compute the standard deviation.

The following formulas were used to compute LoD and LoQ:

Equation I

$$LOD = \Phi_{n,\alpha} \cdot \frac{s_L}{b}$$

Equation II

$$LOQ = k \cdot \Phi_{n,\alpha} \cdot \frac{s_L}{d}$$

Where s_L is the standard deviation of the ten replicates and the factor $\Phi_{n,\alpha}$ take into account the probability that certain response could be due to the standard deviation of the blank rather than the one of the analyte. The factor k corresponds to the reciprocal value of the desired accuracy.

For 10 measurements and at a 95% confidence level (α = 0.05) the $\Phi_{n,\alpha}$ factor is equal to 1.9. LOQ is computed using a k factor of 2, which give a 50% of accuracy. From this, the following computation results:

LOD = 0.8 µg/L

LOQ= 1.6 µg/L

4.8.3 Trueness

Trueness was calculated using the standard addition method, as CRMs were not available. Spiked samples were handled as specified in Subsection 4.7.4.

Two concentration levels of spiking were used and samples were analysed in triplicate for five different days. To compute the recovery rates at each concentration level, three samples were prepared: real sample, real sample with standard addition (*level 1*) and real sample with double standard addition (*level 2*). The amounts of silver standard added to the real sample of NM-300 RMN

dispersion using a pipette, were respectively 8 µl and 16 µl; however, for more accurate determination at these small microliter volumes, a gravimetric approach was used for the quantification of the two aliquots. In Table 7 recovery rates for each day are reported; for the *level 2* on the 5th day, one replicate was rejected because of a suspected loss of sample during the rinse procedure after mineralization. The average recovery rate is 99%.

Table 7: Recovery rates.

	day 1	day 2	day 3	day 4	day 5
level 1	100%	105%	103%	99%	100%
level 2	95%	100%	93%	98%	98%

4.8.4 Repeatability and intermediate precision

For repeatability and intermediate precision, three samples with different levels of silver concentration (low, medium and high) were measured for 5 different days in three replicates. Results obtained from real and spiked samples in trueness calculation were used.

Repeatability, intermediate precision (or within laboratory reproducibility) and day-to-day variation were evaluated using one-way ANOVA (Analysis of Variance). Results are presented in Table 8 according to silver levels, as precision generally depends on analyte concentration (EURACHEM Guide, 1998).

Table 8: Repeatability and intermediate precision of ICP method.

	low	medium	high
Repeatability	3 %	1 %	2 %
Intermediate precision	3 %	2 %	4 %
Day-to-day	2 %	1 %	1 %

4.8.5 Stability of the Extracts

Sample extracted for trueness study were analysed for a week in order to check their stability. After one week, percentages of recovery do not vary significantly.

4.9 Estimation of the measurement uncertainty

The estimation of the measurement uncertainty was performed using the method expressed in the EURACHEM/CITAC Guide (Ellison *et al.*, 2000).

The aim of this uncertainty assessment was to provide the expanded uncertainty associated with the measurement of silver content NM-300 material by ICP-OES techniques, after the microwave-assisted digestion procedure. In order to analyse each source of error the cause-effect diagram was designed (Figure 5). The combined uncertainty was computed using the propagation error law and the expanded uncertainty was obtained from the preceding one by multiplication of a coverage factor, *k*, which takes into account the confidence limit (Ellison *et al.*, 2000).
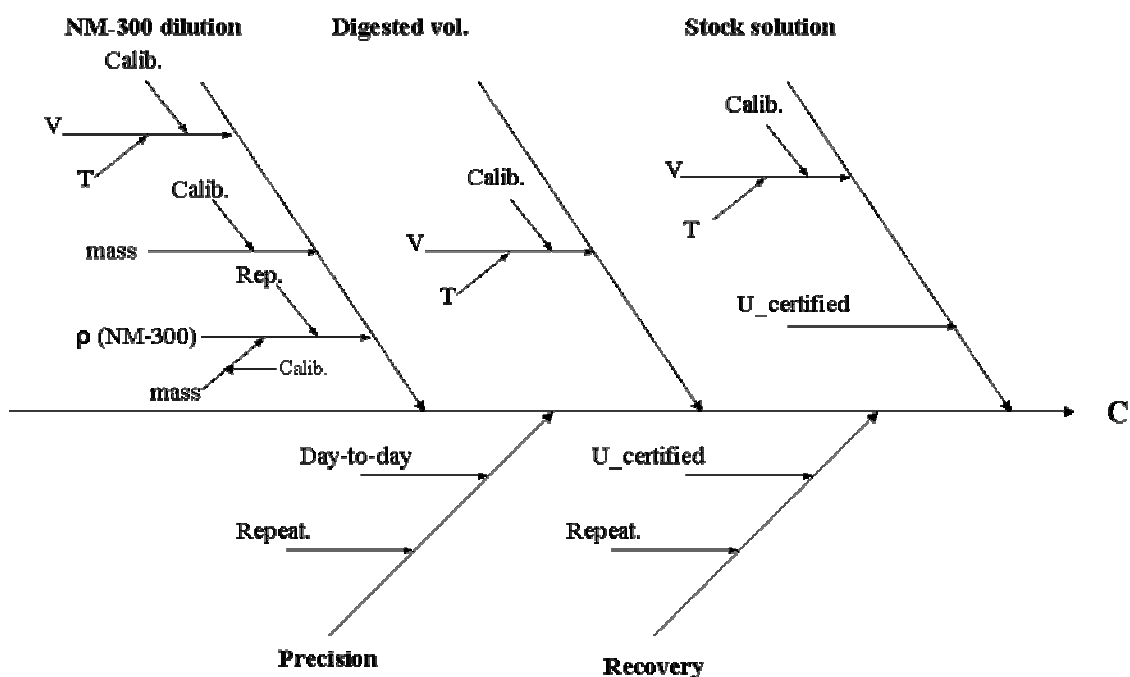
Figure 5: Cause-effect (or Hishikawa) diagram used for uncertainty assessment.

4.9.1 Combined Uncertainty

The concentration of the total silver content in each sample, obtained after the mineralization procedure and ICP-OES analysis, as described in Subsection 4.7, was derived from the following equation:

Equation III

$$C = C_{ICP} \cdot d_1 \cdot d_2$$

C_{ICP} is the value, in mg/L, derived from ICP-OES analysis, d_1 and d_2 are respectively the diluting factors before and after the mineralization process, expressed by:

Equation IV

$$d_1 = V_1 \cdot \rho / m_{NM300}$$

Equation V

$$d_2 = V_2 / \text{pipette}$$

with m_{NM300} and ρ being the mass used for dilution and the density of NM-300 material, *pipette* the volume of diluted NM-300 solution used for mineralization process, V_1 and V_2 the diluting volumes. Based on the cause-effect diagram, the main factors that contribute to the overall uncertainty were found to be the method recovery, precision, concentration of diluted standard stock solution and NM-300, and the final volume of sample digest. Starting from the contribution of the single uncertainties and using the error propagation low the combined uncertainty, expressed in terms of relative uncertainties u_i, can be calculated using the following equation:

Equation VI

$$u_{rel}(C) = \sqrt{u_{rel}^2(\text{stock}) + u_{rel}^2(\text{NM} - 300) + u_{rel}^2(\text{Vfinal}) + u_{rel}^2(\text{rec}) + u_{rel}^2(\text{precision})}$$

In the next sections all contributions described above are analysed individually. The uncertainty caused by pipetting operations was taken into account in precision studies, since different fixed and adjustable-volume pipettes were used during the measurements of NM-300.

4.9.1.1 Silver Standard Stock Solution

The uncertainty associated with the silver standard stock solution used for calibration is a combination of the uncertainty associated with the silver-

concentration uncertainty, given in the certificate of the solution, and uncertainty derived from the volumetric flask used for dilution.

The certificate standard uncertainty (given by the manufacturer) of the silver stock solution is equal to 1000 ± 2 mg/l. As this value is not correlated with a confidence level or distribution information, a rectangular distribution is assumed and the uncertainty is divided by $\sqrt{3}$.

Equation VII

$$u_{cert} = \frac{2}{\sqrt{3}} = 1.15 \quad mg/l$$

The uncertainty of the100 ml volumetric flask used for diluting the stock solution was computer combining the uncertainties arising from temperature and calibration effects.

The tolerance of the 100 ml volumetric flask (given by the manufacturer) is set to 0.1 ml at a temperature of 20 ºC. As no confidence level is reported, a triangular distribution was assumed and the uncertainty associate with calibration effect is:

Equation VIII

$$u_{calib} = \frac{0.1}{\sqrt{6}} = 0.04 \quad ml$$

In order to account for the temperature variability in the laboratory within ±3 ºC respects to the calibrating temperature (20ºC), a rectangular distribution was assumed and the uncertainty associate to this effect is computed with the following formula:

Equation IX

$$u_{temp} = \frac{T \cdot V \cdot Q}{\sqrt{3}} = 0.04 \quad ml$$

Where T is the temperature variability (±3), V is the volume of the volumetric flask used and Q is the coefficient of volume expansion of the water (Q = 2.1 x 10^{-4} ºC^{-1}).

The combined uncertainty of the 100 ml volumetric flask is:

Equation X

$$u_{volum} = \sqrt{u_{calib}^2 + u_{temp}^2} = 0.05 \quad ml$$

The combined uncertainty of the silver stock solution was calculated combining the three uncertainties, see Table 9.

Table 9: Combined uncertainty of silver stock solution.

Description	Value	SD	Uncertainty as RSD (%)
silver stock solution (u_{cert})	1000 mg/l	1.15 mg/l	0.12
Volumetric flask (u_{volum})	100 ml	0.05 ml	0.05
Combined uncertainty (u_{stock})			0.13

Equation XI

$$\frac{u_{stock}}{C_{stock}} = \sqrt{\left(\frac{u_{std}}{C_{stock}}\right)^2 + \left(\frac{u_{volum}}{V}\right)^2} = 0.13 \quad \%$$

4.9.1.2 NM-300 Diluted Solution

The uncertainty associate to the NM-300 diluted solution used for testing is a combination of the uncertainties arising from the volumetric flask, the NM-300 mass and the solution density (ρ). The uncertainty associated with the silver concentration in NM-300 solution is not known.

The volumetric flasks used for NM-300 dilution were the same as those used for the silver stock solution, hence the uncertainty associated with the volumetric flask is the same.

The contribution from the weight of NM-300 taken for dilution is due to the linearity uncertainty of the balance from Calibration Certificate. From balance linearity (± 0.03 mg), a rectangular distribution is assumed to obtain a standard uncertainty; this contribution is considered twice, once for the tare and once for

the gross weight. This gives of the following value of the standard uncertainty of NM-300 mass, u_m,:

Equation XII

$$u_m = \sqrt{2 \cdot \left(\frac{0.03}{\sqrt{3}}\right)^2} = 0.02 \quad mg$$

The amount of NM-300 dispersion, which was used in the experiments, was approximately identical, measured and described in mass determined by weighing. In order to calculate the relative standard deviation, the mean mass weight (55 mg) was used as point of reference.

NM-300 density was computed according to the procedure described in Subsection 4.7: Density Computation and associated uncertainty is based on both the standard uncertainty due to repeated measurements, u_{rep}, and the NM-300 mass. The uncertainty associated with NM-300 solution weight is u_m. The uncertainty due to NM-300 density can be calculated by combining u_m and u_{rep}:

Equation XIII

$$\frac{u_\rho}{\rho} = \sqrt{\left(\frac{u_m}{mass}\right)^2 + \left(\frac{u_{rep}}{\rho}\right)^2} = 2.88 \quad \%$$

The combined uncertainty of the NM-300 diluted solution was calculated combining the two uncertainties above (see Table 10):

Table 10: Combined uncertainty of NM-300 diluted solution.

Description	Value	SD	Uncertainty as RSD (%)
Volumetric flask (u_{volum})	100 ml	0.05 ml	0.05
Mass (u_m)	55 mg	0.02 mg	0.04
Density (u_ρ)			2.88
Combined uncertainty (u_{NM300})			2.88

Equation XIV

$$\frac{u_{NM-300}}{C_{NM-300}} = \sqrt{\left(\frac{u_{volum}}{V}\right)^2 + \left(\frac{u_{density}}{\rho}\right)^2} = 2.88 \quad \%$$

4.9.1.3 Final Digested Volume

This uncertainty is due only to the 50 ml volumetric flask used to collect the sample after the microwave digestion process.

As in the previous cases the uncertainty associated to the volumetric flask is a combination of the calibration and temperature effects.

Equation XV

$$u_{Vfinal} = \sqrt{u_{calib}^2 + u_{temp}^2} = 0.04 \quad ml$$

Uncertainty as relative standard deviation is reported in Table 11.

Table 11: Uncertainty of volumetric flask for final digested volume.

Description	Value	SD	Uncertainty as RSD (%)
Volumetric flask (u_{Vfinal})	50 ml	0.04 ml	0.09

4.9.1.4 Recovery

The overall bias of the analytical method is determined by the recovery study of the method validation procedure, using the standard addition method.

The uncertainty due to recovery is derived from the standard deviation of the mean of the trueness assessment study (u_{tr}) and from the uncertainty associated to the analytical standard used for spiking, as described in the following equation (see Table 12):

Equation XVI

$$u_{rec} = \sqrt{\frac{s_{tr}^2}{n_{tr}} + u_{std}^2}$$

where s_{tr} is the relative standard deviation derived from the average recovery of each day, equal to 0.02, and n_{tr} = 5 is the number of days.

The uncertainty associated with the silver standard was previously estimated, assuming a rectangular distribution.

Table 12: Combined uncertainty from recovery test.

Description	Value	SD	Uncertainty as RSD (%)
Trueness (u_{tr})			0.88
Ag standard (u_{cert})	1000 mg/l	1.15 mg/l	0.12
Combined uncertainty (u_{rec})			0.89

A t-test was computed in order to determine whether the mean recovery ($\overline{R}_m$) is significantly different from 1. The following equation was used:

Equation XVII

$$t = \frac{\left|1 - \overline{R}_m\right|}{u_{tr}}$$

The t value obtained was compared with the critical value, t_{crit}, with *n-1* degree of freedom at 95% confidence level (Ellison *et al.*, 2000), where *n* is the number of results used to calculate the average recovery. Average recovery is significantly different from 1 if $t \geq t_{crit}$. In this case it results $t = 1.16 < t_{crit} = 2.04$ and hence no correction factor is to be applied to recovery
It results that the average recovery is not significantly different from 1 ($t = 1.16 < t_{crit} = 2.04$) and hence no correction factor is to be applied.

4.9.1.5 Precision
Uncertainty associated with precision was derived from two contributions: repeatability and intermediate precision calculated in the validation study.

Uncertainty due to repeatability was estimated as $s_{rep}\big/\sqrt{n_{rep}}$ where s_{rep} is relative standard deviation due to repeatability experiment and n_{rep} the number of replicates.

The uncertainty due to intermediate precision was estimated as $s_{day}\big/\sqrt{d}$ with s_d being the relative day-to-day variation and d the number of days.

The precision uncertainty was derived combining these two uncertainties:

Equation XVIII

$$u_{prec} = \sqrt{u_{rep}^2 + u_{day}^2}$$

As precision varies with varying concentration level, three levels of uncertainty have been computed (Table 13), associated to the concentration level from which they are derived from (low, medium, high).

Table 13: Uncertainty due to precision at three concentration levels.

Description	RSD (%) Low	RSD (%) Medium	RSD (%) High
Repeatability (u_{rep})	0.67	0.49	1.03
Intermediate precision (u_{day})	0.99	0.72	0.67
Combined uncertainty (u_{prec})	1.19	0.87	1.23

Starting from the contribution of the single uncertainties, the combined uncertainty, expressed in terms of relative uncertainties u_i, can be calculated using Equation VI. In Table 14 all the contributions are reported.

Table 14: Relative standard deviation contributions for combined uncertainty.

Description	Uncertainty as RSD (%)
Stock solution (u_{stock})	0.13
NM-300 (u_{NM-300})	2.88
Final Vol. (u_{Vfinal})	0.09
Recovery (u_{rec})	0.089
Precision (u_{prec})	0.87-1.23

From Figure 6, the major contributions to the uncertainty budget were found to be NM-300 diluting factor, in which density uncertainty is predominant, method recovery and precision. The other two contributions were insignificant.
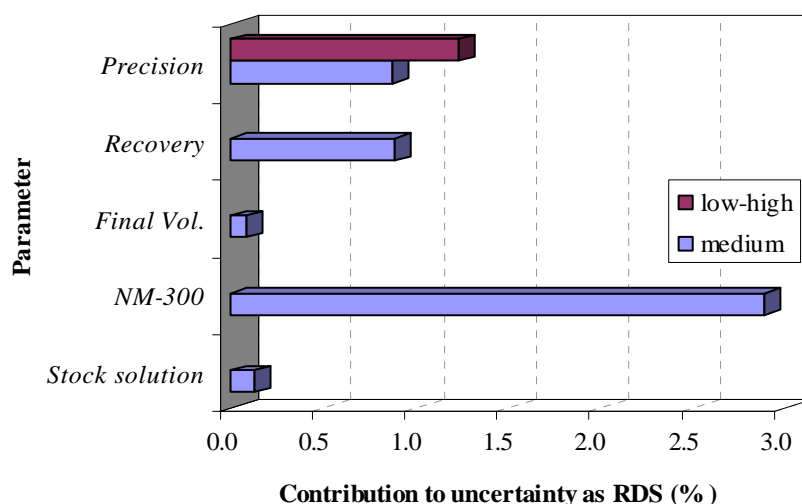
Figure 6: Major contributions to uncertainty.

The combined uncertainty was computed for two level of precision, low-high and medium, resulting slightly different for the two different levels:

Equation XIX

$$u_{combined} = 0.033 \ \text{(medium)} - 0.034 \ \text{(low} - \text{high)}$$

In percentage terms the combined uncertainty is equal to **3.3 – 3.4%**.

4.9.2 Expanded uncertainty

In order to take into account a confidence level, the combined uncertainty is to be multiplied by a coverage factor, *k*, to produce the expanded uncertainty.

The choice of this factor was done taking into account a 95% confidence level, which give a coverage factor of 2. The expanded uncertainty, equal for both level of precision, is given by:

Equation XX

$$u_{expanded} = k \cdot u_{combined} = 0.07$$

In terms of percentage, the assessment of uncertainty associated with the NM-300 silver solution measurement with ICP-OES method and microwave assisted digestion, gives an **expanded uncertainty of 7%.**

5 Results and discussion: Characterisation, stability and homogeneity

5.1 Transmission electron microscopy (TEM): Characterisation

5.1.1 IUTA results

NM-300 RMN with sample identification number "**0121**" was used. Overall 2236 particles were measured with TEM analysis using an average magnification of 66,000. The image resolution is 1024×1024 pixel. At this magnification, a pixel corresponds to a length of 0.411 nm, which is the highest possible accuracy of the analysis.

The pictures used in the analysis are shown in the Appendix A. In Figure 7, two high resolution pictures for a detailed view are reported.
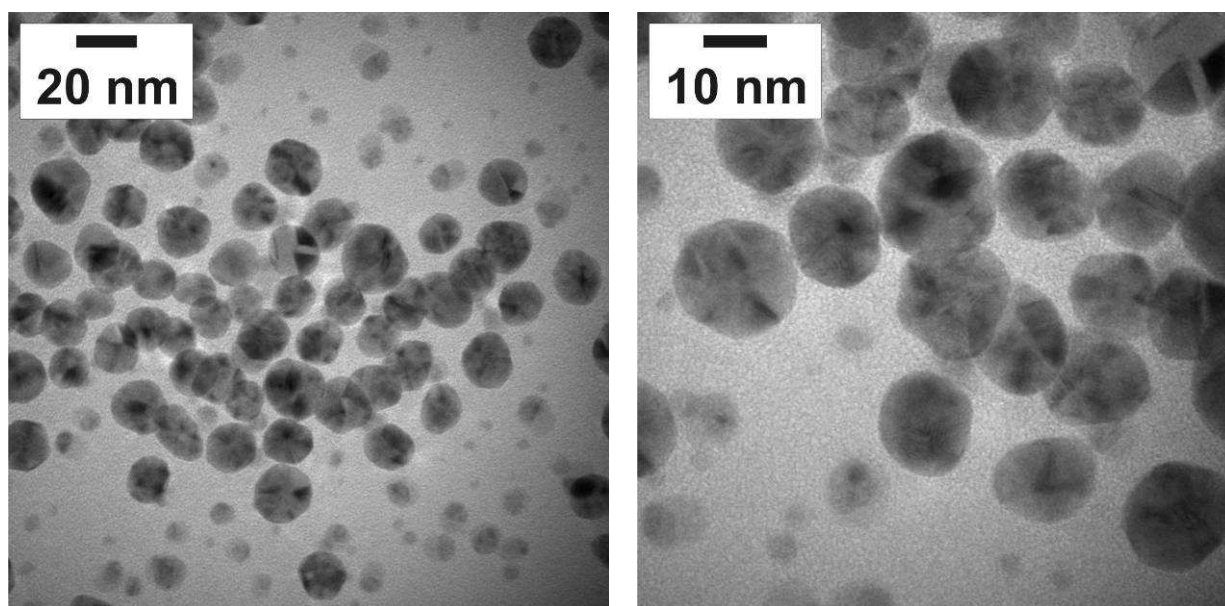
Figure 7: EM characterization of NM-300 material at different levels of magnification.

As shown in Figure 7 (particularly at higher magnification), the particles are crystalline and faceted. In some cases, the icosahedron structure is clearly observable. Obviously, the particles are not coated with an oxide layer, and show a small amount of self-organisation on the applied TEM grids.

Statistical analysis reveals two modal diameters, which are observable in the particle size distribution of the total measurement (Figure 8).
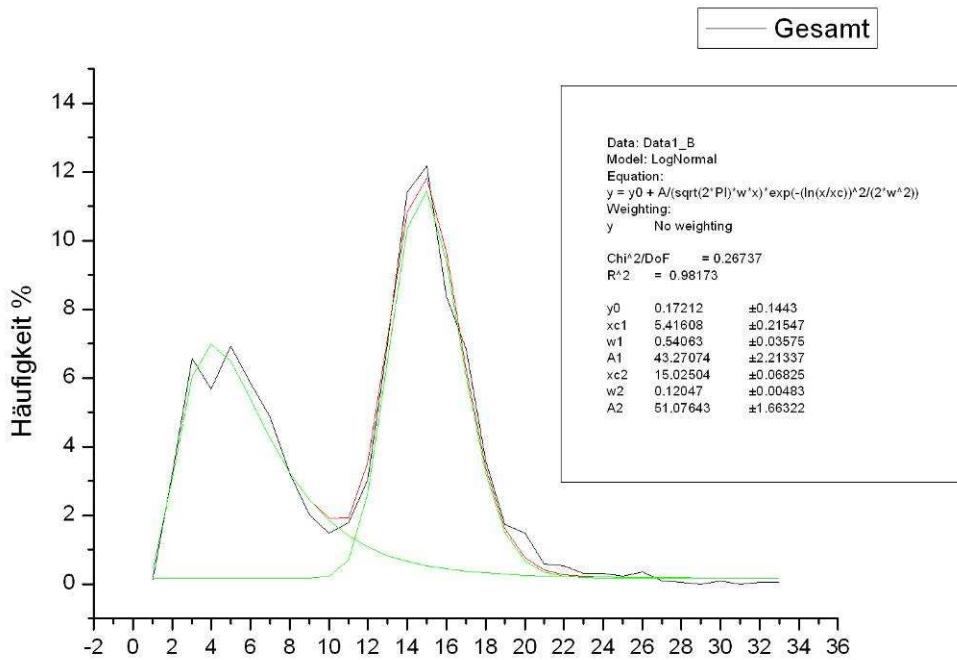
Figure 8: Measured size distribution and Origin-fit of the total TEM dataset.

The size distribution of the total measurement and the individual pictures were fitted by an Origin multi-peak lognormal distribution. The resulting parameters are listed in Table 15.

Table 15: Results of the fit of the TEM distributions.

	Raw Data		Origin Fit				
TEM	Number (N)	Max. diameter (nm)	Diam. 1 (nm)	σ 1 (nm)	Diam. 2 (nm)	σ 2 (nm)	R²
Pic. a	571	25.66	5.36	1.82	14.93	1.12	0.96
Pic. b	578	31.96	5.54	1.58	15.14	1.14	0.97
Pic. c	515	27.51	5.27	1.75	14.86	1.12	0.93
Pic. d	572	32.59	5.53	1.70	15.11	1.13	0.97
average			**5.43**	**1.72**	**15.01**	**1.13**	**0.96**
Pics a-d combined	**2236**	**32.59**	**5.42**	**1.72**	**15.03**	**1.13**	**0.98**

The modal diameters of the two individual peaks of the total dataset amount to *Mode1* = 5.42 nm and *Mode2* = 15.03 nm, respectively. The average of the two modal diameters of the four individual pictures amount to *Mode1* = 5.43 nm and *Mode2* = 15.01 nm, respectively.

5.1.2 TEM analysis by VAR

NM-300 vial with sample identification number "**0206**" was used. The samples were brought on home-made pioloform- and carbon-coated EM-grids pretreated with Alcian Blue 1%, and air dried. For comparison, a sample without Alcian Blue pre-treatment was examined also.

Magnification was initially set at 30,000x and subsequently was set at 68,000x in order to characterized silver nanoparticles of smaller diameters. To obtain quantitative results, micrographs were analyzed using the Analysis Solution of the iTEM software (Olympus, Münster, Germany).

A manual threshold method was applied. Particles were detected in a pre-defined frame (region of interest) excluding border particles and particles consisting of less than 150, for a magnification of 30,000, and less than 50 pixels for a magnification of 68,000. Possible holes within the particles are eliminated with the 'Fill holes' command.

For each particle the following parameters were measured: Particle ID, Class ID, Area, Convex Area, Convex Perimeter (nm), Convexity, Elongation, Shape Factor, Sphericity, Orient. Aspect Ratio, Diameter Max (nm), Diameter Mean (nm), Diameter Min (nm), Aspect Ratio, ECD, Next Neighbor ID, Perimeter, Rectangle Max, Rectangle Mean and Rectangle Min.

5.1.2.1 Analysis at a Level of Magnification of 30,000x

In Figure 9, a micrograph taken from an Alcian Blue treated grid is reported. As expected, the grid without Alcian blue contained less particles than the grid with Alcian blue (approximately 5 times less). However, both contained more than enough particles to perform an acceptable analysis.
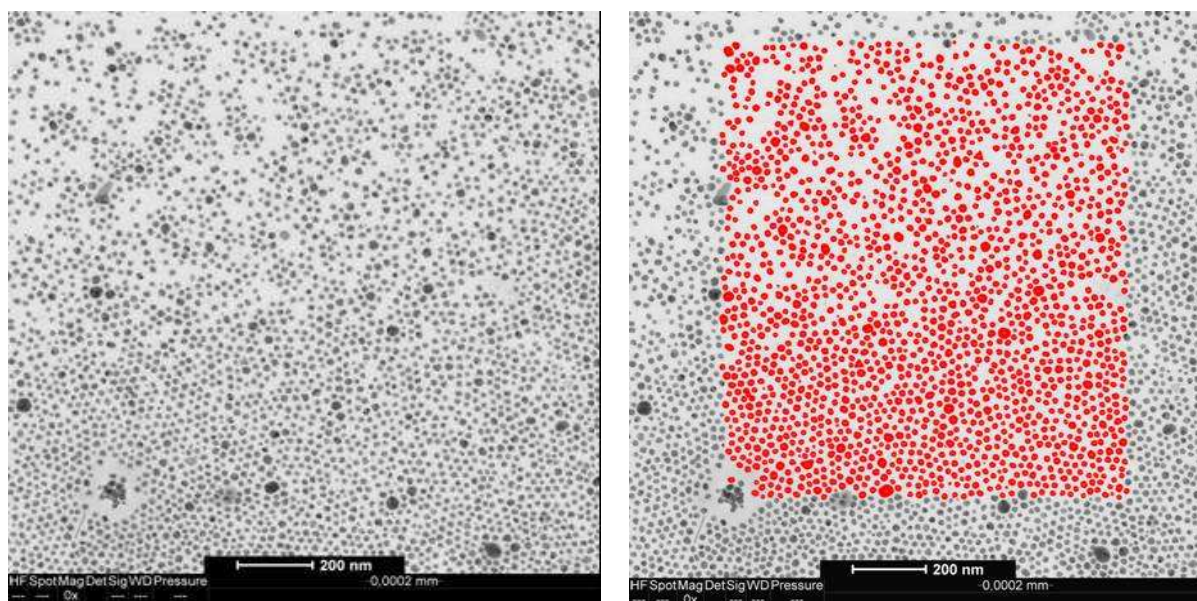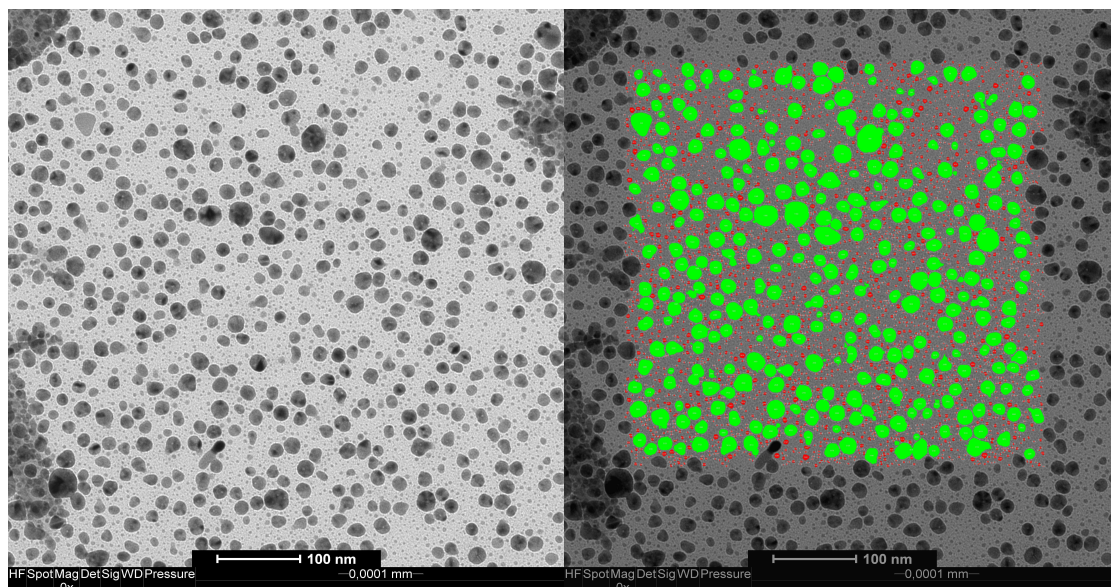
Figure 9: Representative micrograph (left) and the corresponding annotated micrograph (right). These are micrographs taken from an Alcian Blue treated grid.

Table 16 reports a number of particles analysed on four micrographs from grids which were treated with Alcian blue and on two micrographs from grids which were not treated with Alcian blue and mean diameters.

Table 16: Number of particles and mean diameter of NM-300 with and without Alcian Blue grid treatment.

	number of particles				mean diam (nm) calculated
	microg. 1	*microg. 2*	*microg. 3*	*microg. 4*	
with Alcian Blue	850	1756	1872	1398	17.256
without Alcian Blue	268	348			17.251

No difference in mean diameter was detected between particles attaching to grids treated with Alcian Blue (17,256 nm) or without Alcian Blue (17,251 nm). In Figure 10 (magnification 30,000x), the number distribution of the mean diameter of NM-300 from one of the analysed micrographs is reported.
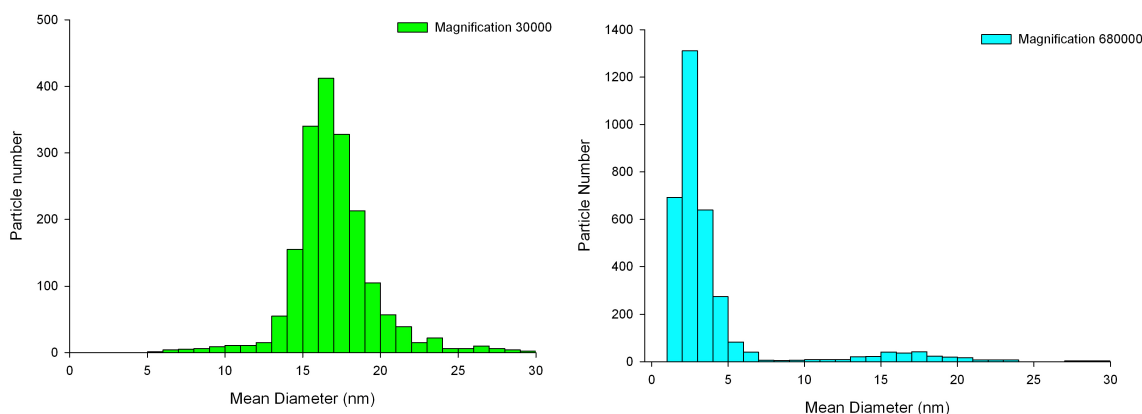
Figure 10: **Number distributions of the mean diameter of the silver-nanoparticles (NM-300) from one of the analysed micrographs taken at different original magnifications. To distinguish the small particles from the background a higher magnification was required.**

A simple inspection demonstrated different shapes of particles within the sample. The majority of the particles have a somewhat round shape; others are triangular or trapezium-like. iTEM software allows to classify (and selectively analyze) particles according to different measurements including size and shape. For example, in Figure 11, only needle-like or triangular shaped particles were selected.

Particles of a specific shape can be detected semi-automatically based on a combination of certain measurements. In example, for the triangles the following parameters have been selected: Convexity, Elongation, Shape Factor, Aspect Ratio and Orientation Aspect to be able to sort them out. Needle-like structures need less parameters to be able to select them: elongation will be sufficient to sort these particles out.
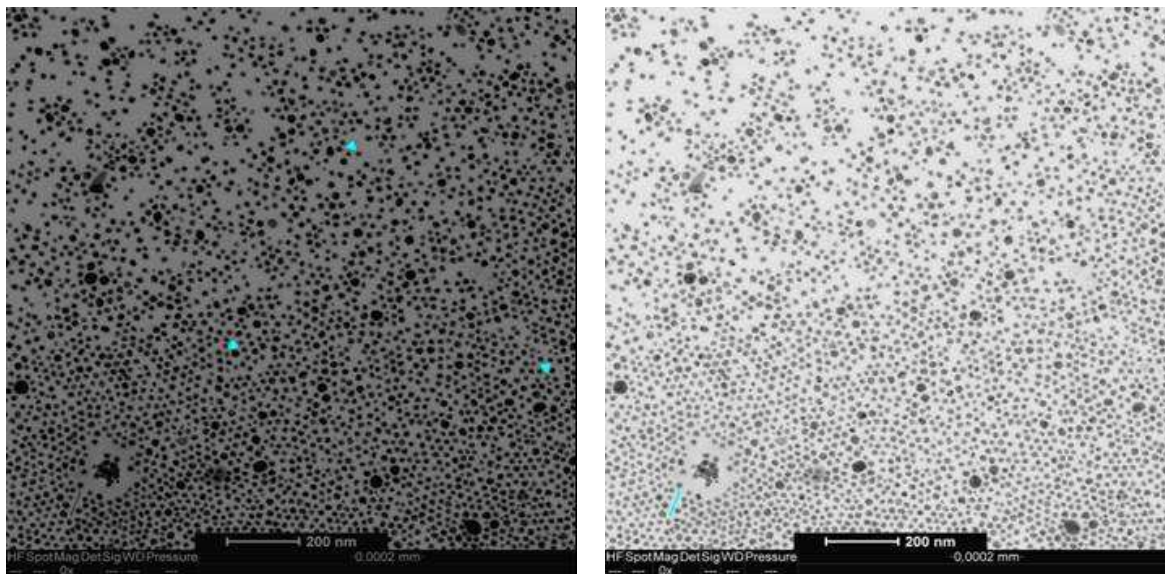
Figure 11: Micrograh showing only particles of a specific shape as detected by the iTEM software. In the micrograph on the left only triangles are detected. On the right, only needle shaped particle(s) were detected.

In addition to the majority of bigger particles of about 20 nm, there are some smaller particles present in the sample. These small particles were excluded from analyses at this magnification level.

5.1.2.2 Analysis at a Level of Magnification of 68,000x

This level of magnification was used in order to characterize silver nanoparticles with a mean diameter of 3-4 nm. Number distributions of the mean diameter of NM-300 from one of the analysed micrograph are presented in Figure 7 (magnification at 68,000x).

In **Figure 12**, one of the micrographs analysed for the characterization of smaller silver nanoparticles; the other two micrographs are reported in Appendix B. An arbitrarily set group classification has been used to divide the particles in two groups: the first group with a mean diameter of 1-8 nm and the second one with a mean diameter of 8-100 nm, which was already with a magnification of 30,000. Three thousand three hundred and fifty two, 3093 and 2152 particles were analyzed.

Figure 12: Representative micrograph (left) and the corresponding annotated micrograph (right). These are micrographs taken from an Alcian Blue treated grid. On the micrograph on the right, gray values have been modified, to obtain a better analysis.

5.1.3 Electron Microscopical Analysis by EMPA

TEM grids (400 mesh, coated with 8 nm of carbon) were incubated for 20 s on a 10 µl droplet of NM-300 Silver suspension diluted 1:90 with nanopure water. Subsequently the excess suspension fluid was drawn off with a filter paper and the grids were imaged in the instrument. Images with the size of 1024 x 1024 pixels and a pixel size representing 0.376 nm were analysed with the image processing software IPS Version 1.123 (Visometrics, Konstanz, Germany) to determine the particle size of silver nanoparticle in the suspension. In total 649 particles were analysed.

All these measurement results are plotted as histogram and density plot (see Figure 13) using R software (http://www.r-project.org). At the same time the mean, standard deviation and their robust counterparts (median and MAD) of the size measurement was determined (see Table 17).

Table 17: Statistical parameters evaluated from size measurement analysis.

| Mean	Std.dev.
17.24 nm	3.17 nm
	18.4 %

Median	MAD
16.89 nm	1.90 nm
	11.2 %
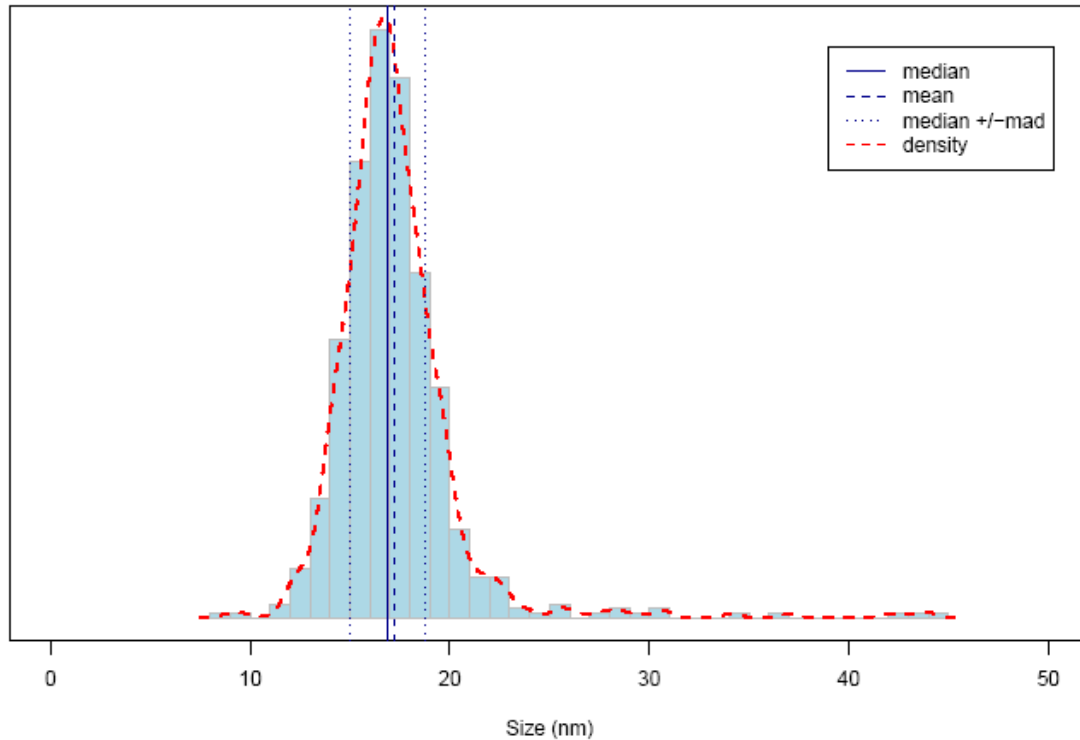
Figure 13: Histogram and density plot of TEM measurement.

The small difference between the mean and median value shows that the particle size has tentative similarities to a symmetric normal distribution.

5.2 Scanning Electron Microscopy (SEM) Characterisation

NM-300 material with sample identification number "**0121**" was used by IUTA for SEM analysis. A total of 2429 particles were measured in eight micrographs. Six of them were made with a magnification of 500,000, two with a magnification of 250,000. The image resolution is 1024 times 728 pixels. A pixel corresponds to a length of 0.68 nm, which is the highest possible accuracy of the analysis.

As the SEM images are out of focus, the blurred particle edges are expected to cause a measurement error in the analysis.

The figures used in the analysis can be found in the Appendix C.

A different, semi-automatic procedure was used for the analysis of the SEM images. This procedure is also described in the Appendix C.

As particles with a diameter of less than 6.3 nm were not regarded in the analysis, the size distribution has only one peak and is therefore approximated by an Origin single-peak lognormal distribution (Figure 14).
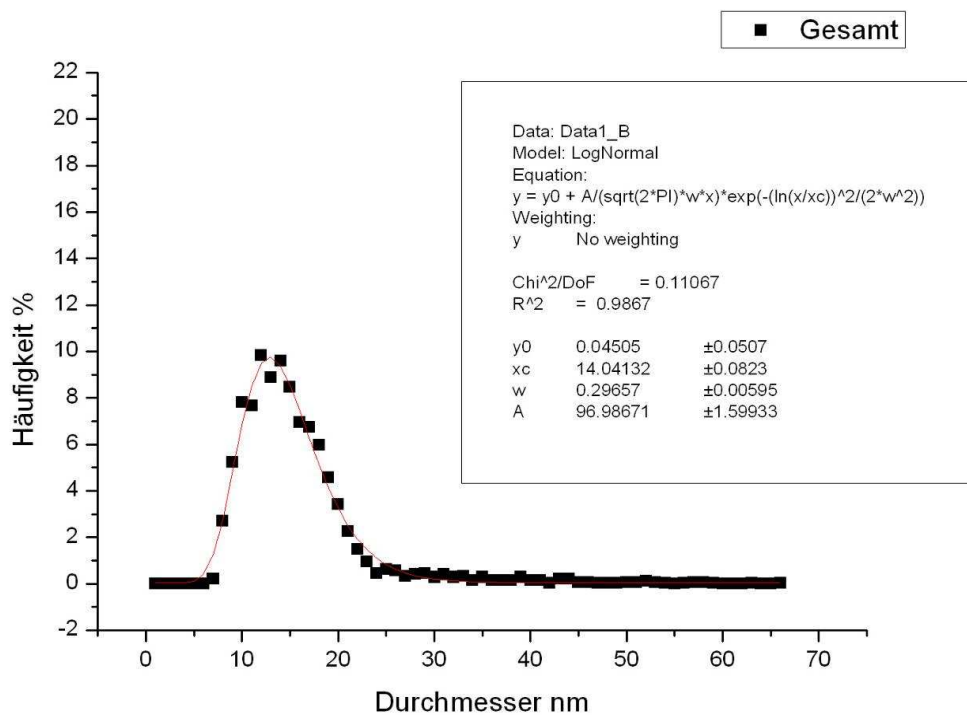
Figure 14: Measured size distribution and Origin-fit of the total SEM dataset.

The resulting parameters are listed in Table 18.

Table 18: Results of the fit of the SEM distributions.

SEM	Raw Data		Origin Fit		
	Number (N)	Max. diameter (nm)	Diameter (nm)	σ (nm)	R²
Pic. 13	428	46.37	17.02	1.21	0.93
Pic. 14	256	58.44	14.95	1.31	0.92
Pic. 15	336	59.29	16.59	1.30	0.94
Pic. 16	304	43.41	14.03	1.27	0.97
Pic. 18	411	33.1	12.96	1.23	0.98
Pic. 19	217	21.01	11.83	1.22	0.94
Pic. 20	166	51.6	10.76	1.28	0.83
Pic. 21	311	65.96	11.3	1.27	0.94
average			13.68	1.26	0.93
Pics **13-21** combined	2429	65.96	14.04	1.35	0.99

The mode diameter here is 14.04 nm for the combined data set, while the average of the individual pictures amounts to modal diameter of 13.68 nm. This means a deviation of 6.6% and 8.9%, respectively, from the TEM values for *mode2*.

5.3 Size distribution by Nanoparticle Tracking Analysis (NTA), by Dynamic Light Scattering and Zeta-Sizer

5.3.1 Nanoparticle Tracking Analysis (NTA)

Six consecutive measurements with freshly made solutions of the NM-300 nano-Silver dispersion diluted 1:1000 with nanopure water have been done with NTA by EMPA. The obtained particle sizes were plotted as histogram and density distribution (see Figure 15), using R, and mean, standard deviation, median and MAD values have been determined (Table 19).

Table 19: Statistical parameters evaluated from size measurement analysis.

Mean	Std.dev.
52.31 nm	25.99 nm
	49.7 %

Median	MAD
47 nm	17.79 nm
	37.9 %
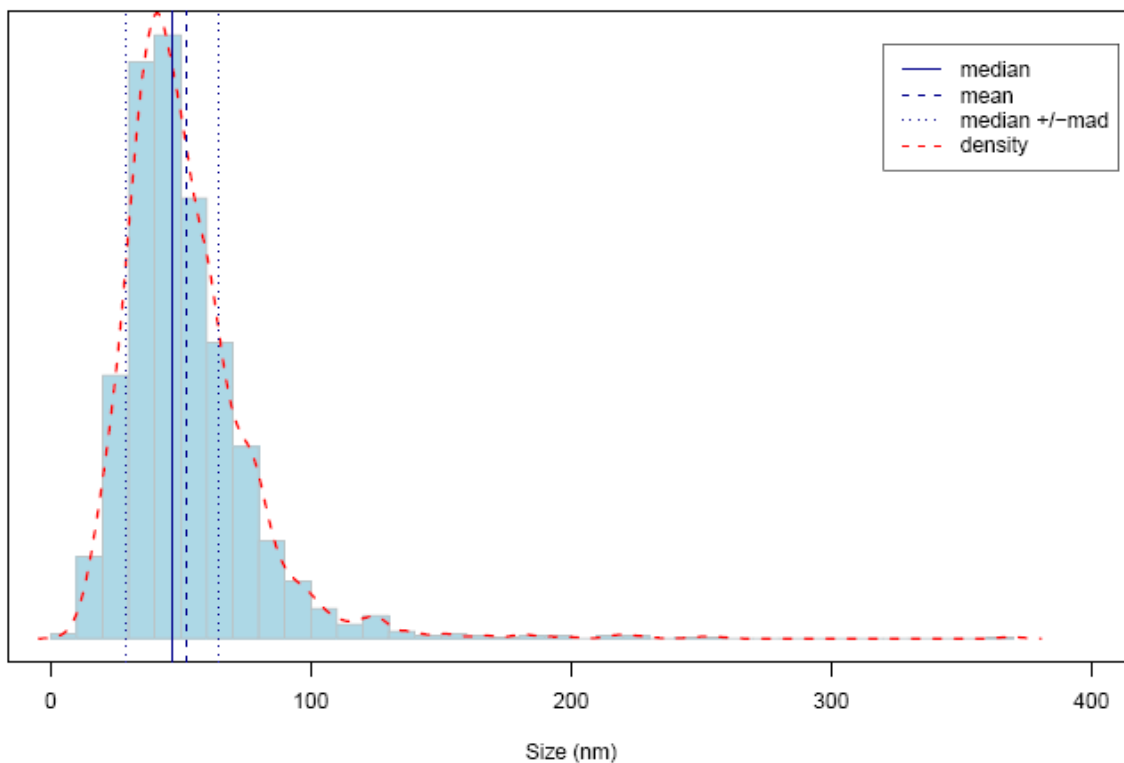
Figure 15: Histogram and density plot of NTA measurement.

The considerable difference of 5 nm between the median and the mean value shows that the size distribution is not perfectly symmetrical. Figure 15 reveals an asymmetric distribution with more large size particles compared to the result obtained with TEM The symmetric distribution of the particle size is concordant for the findings of all participating laboratories. The observed tailing for the NTA results therefore gives a strong indication that there are limitations of the performance, which has a significant impact on the usability, appropriateness of the methodology and interpretation of results.

5.3.2 Dynamic Light Scattering, Zeta-sizer analysis

The NM-300 has been used in a number of collaborative projects under funding schemes of the Framework Programme 7 of the European Commission or national funding schemes of the Member States of the European Union. Several laboratories have reported about their findings, which are published elsewhere. Measurements of particle size using Dynamic Light Scattering (DLS) were shown to be dependent on the matrix used for sample preparation. Typical

measurement results for the particle size and size distribution by using DLS indicate mean particle size of around 50 nm or 70 nm. These results may correspond to the hydrodynamic diameter of the particles. It has to be noted that the performance of the method and instrumentation may be limited, if representative nanomaterials are used. Such limitations have been described for measurements of mixtures of particles of different sizes. Although the single components could be measured and the results corresponded to the findings by using TEM, the results regarding the size distribution of mixtures showed significant limitations (Rossi 2010). Size and size distribution determinations by using zeta-sizer analysis resulted in values given at about 100 nm.

As described and discussed at the International Standardisation Organisation under Technical Committee 229 (ISO TC 229) and the Comité Européen de Normalisation TC 352 (CEN TC 352), the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR 2010) as well as in the Guidance Manual for sponsors of the OECD WPMN (OECD 2010) the determination of a property should be addressed by the selection of the appropriate measurand and the corresponding measurement method. An uncertainty budget should be described based on the Guide for Uncertainty in Measurements (GUM). If measurands are addressed, which are different from the measurand and property to be determined, but which may give a hint to a certain property or under ideal conditions may be brought into context and calibrated to provide a meaningful result, the results have to be carefully interpreted, when being taken into account as additional data.

5.4 Stability of NM-300 by UV-VIS analysis

UV-VIS spectra generated for wavelengths 700 nm to 350 nm were compared for NM-300 for samples, which were stored for 12 months. The spectra of samples show no significant differences with regard to the wavelength of maximum absorbance, peak height and width. The wavelength of maximum absorbance corresponds to the nano-Silver particle size and shows no differences over time up to 12 months. The peak height is correlated to the amount of nano-Silver and number of nano-Silver particles in the dispersion. No alterations are observed for the comparisons of samples at 0 months and after 12 months storage. The results were re-confirmed by GF-AAS measurements for total silver content. The half-width of the UV-VIS peak corresponds to the size distribution of nano-Silver particles. Measurement results for samples before storage and after 12 months of storage are statistically not different. The results are shown in Figure 16.
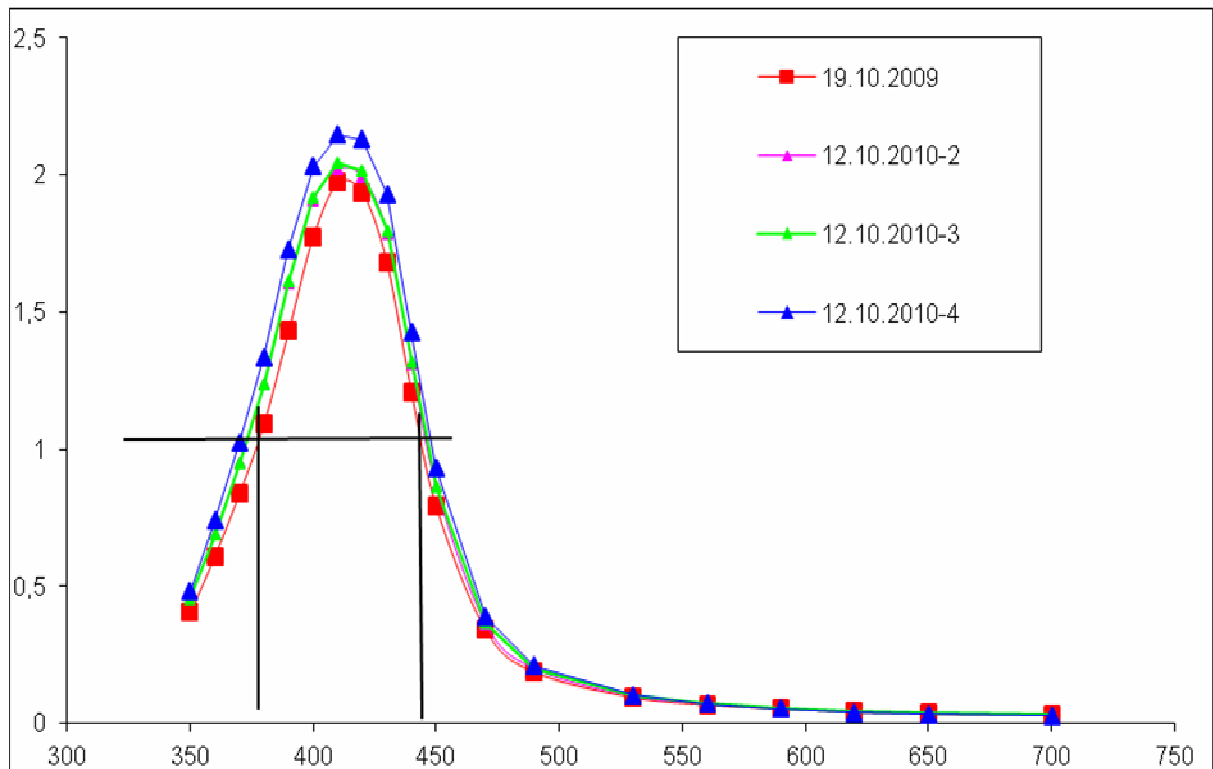
Figure 16: Measured UV-VIS spectra of NM-300 at month 0 and after 12 months of storage.

5.5 Silver ion release from NM-300 embedded in poly-acrylic matrix by GF-AAS

A number of experimental studies were performed regarding NM-300 RMN, which was embedded in a poly-acrylic matrix regarding the elution of silver ions under varying conditions. Strips after preparation were rinsed with double-distilled water and incubated in different media at 37 ℃.

5.5.1 Elution of silver ions in different media

Three media were used: Physiological phosphate buffer, 0.5 % nitric acid and de-ionized water. The release of silver into the elution matrix was observed for up to 13 days without removing and exchanging the elution media. The results are shown in Figure 17. Silver ions are released from the strip into the media. For physiological buffer, after 5 days equilibrium is reached and no further increase of silver concentration in the elution media is observed. In nitric acid, the silver concentration in the elution medium is further increasing over the time of the experiment. The amount released from the strip eluted with nitric acid is about 6000 ng silver. This is threefold the amount released compared to the amount of silver from the stripe eluted by using physiological buffer. The level for the concentration, at which the equilibrium was reached, depended on the silver content of the stripe used.
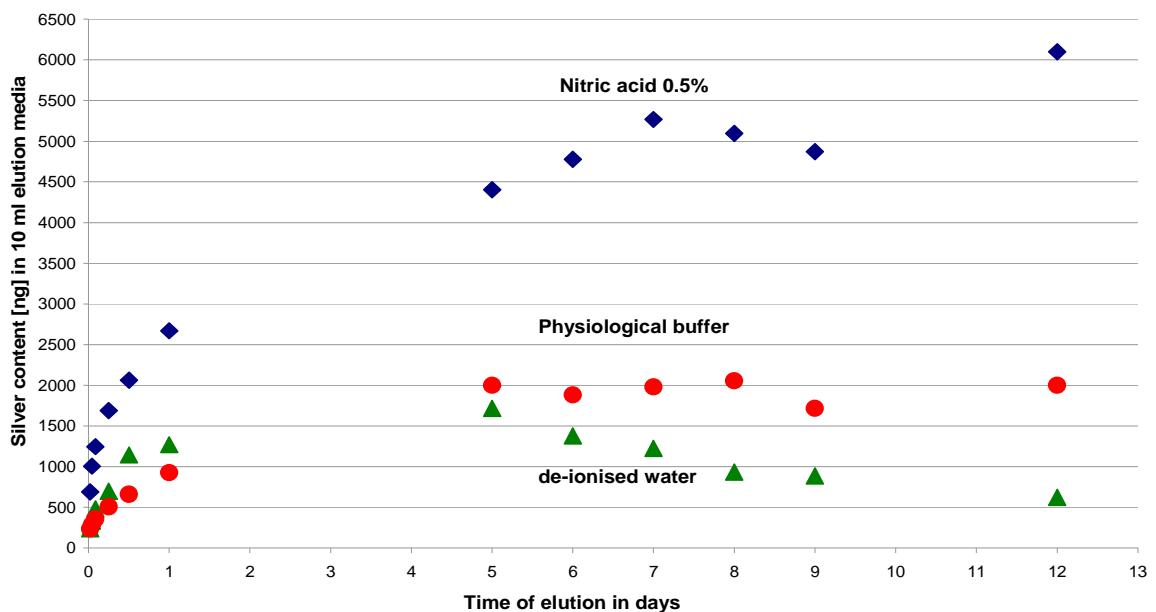
Figure 17: NM-300 embedded in acrylic matrix and eluted by using physiological buffer, 0.5 % nitric acid or deionised water: Silver content in elution media over time in days.

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5.5.2 Silver release from matrix with different content of NM-300

The release of silver into the media was studied depending on the amount of NM-300 embedded into the acrylic matrix, strips, for silver contents from 42 µg/g up to 4000 µg/g. The amount of silver eluted from the strip was correlated to the silver content as illustrated in Figure 18. The release of silver into the matrix as used for NM-300K is reaching an equilibrium at a level of maximum 0.01 % mass of silver being present in the media relative to the total mass of silver in the strip.
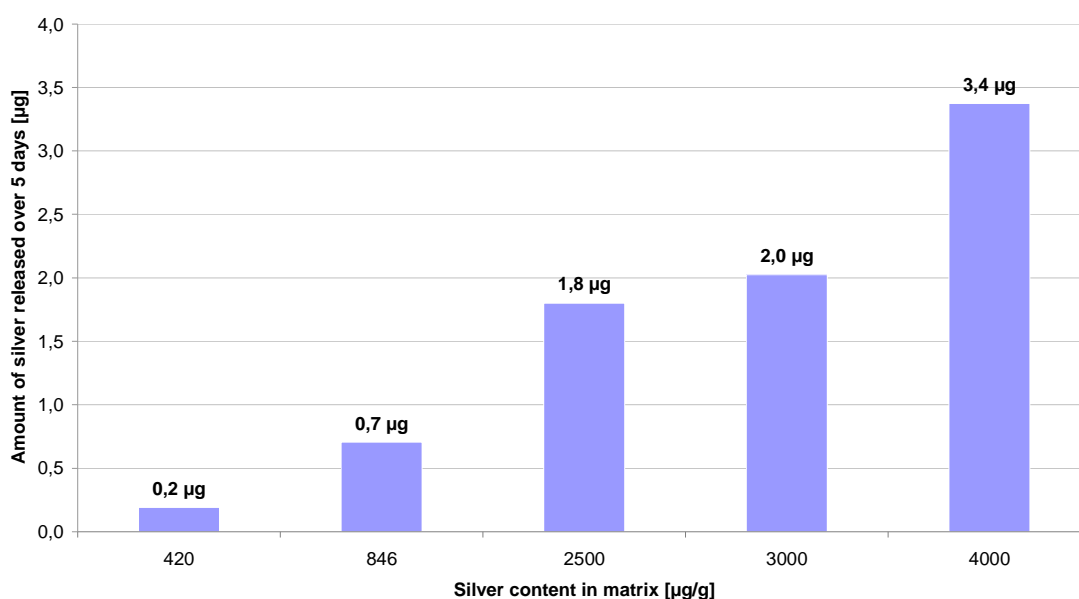
Figure 18: Different amounts of M-300 embedded in acrylic matrix and eluted by using physiological buffer: Silver content in elution media after 5 days.

5.5.3 Forced release of silver from NM-300 in a matrix

The elution of silver from NM-300, which was embedded in a matrix, was examined as for the different types of media, but under forced conditions at 100 ℃ over 5 hours. Equilibrium was found after 1. 5 hours with a silver release of 5.9 µg silver in 10 ml elution media, if physiological buffer and a strip was used, which contained NM-300 resulting in a concentration of 2500 µg/g silver. Further increase of silver concentration was not observed after 1.5 hours.

5.5.4 Long-term elution study with full exchange of elution media

In addition, the release of silver from strips doted with 2500 µg/g was examined for a period of up to 33 days, for which the elution media were exchanged completely after each period of 5 days. The amount of silver determined in the elution media after 5 days from the strip decreases after the second period of incubation, namely after 10 days and further for the time points of determination at days 15, 20 and 25. The quantitative exchange of elution media after 5 days leads to defined levels of silver in the elution media as shown in Figure 19.
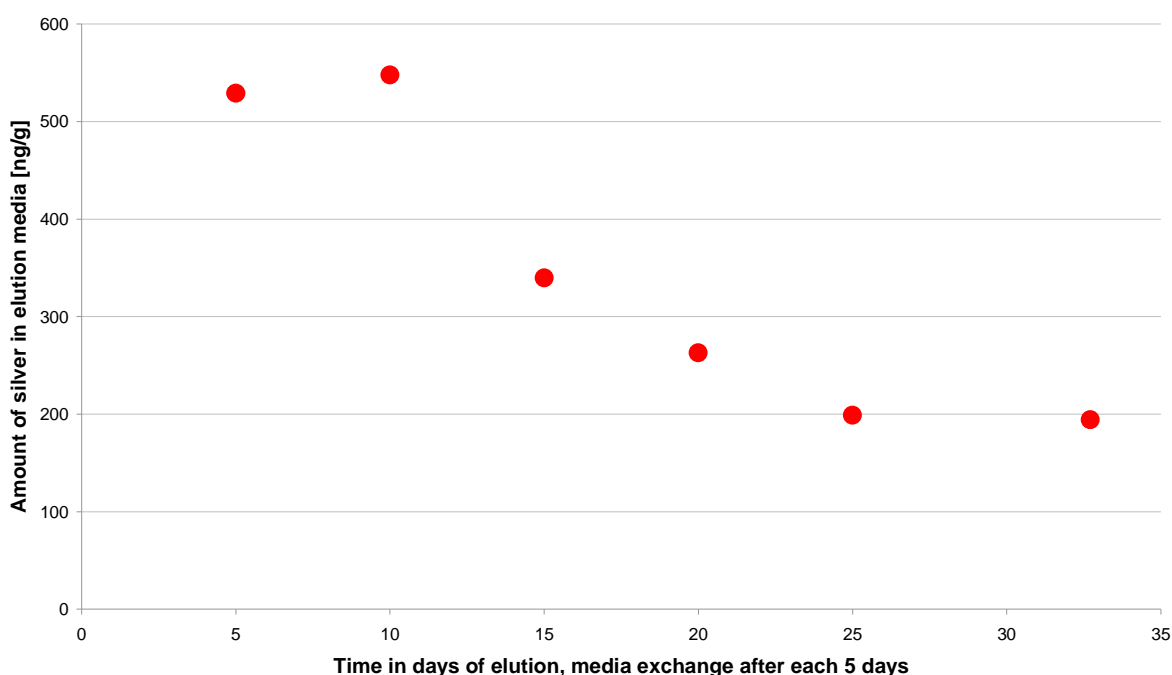
Figure 19: NM-300 embedded in acrylic matrix and eluted by using physiological buffer and quantitative exchange of elution media each 5 days (after each measurement): Silver content in elution media over time in days.

5.6 Homogeneity study by ICP-OES

After the validation of the ICP-OES method for the analysis of NM-300 material and the uncertainty assessment, a homogeneity study was performed in order to confirm the homogeneity of the silver content of the material.

For this experiment 20 units/vials of NM-300/NM-300K material were furnished, which can be grouped in four groups in relation to their origin, as described in Table 20. Additional experiments addressed within bottle homogeneity and the

influence of the conditioning of the vial before sampling including shaking the sample vigorously.

Table 20: NM-300/NM-300K units used and conditioning groups.

	Sample ID	*Description*
Group 1	0030 - 0051 - 0021 - 0033 0075	*Samples from normal production process*
Group 2	0886 - 0897 - 1048 - 1170 1432 - 1468 - 1493	*Samples from normal production process, but different selection compared to Group 1*
Group 3	5061 - 5062 - 5063 - 5064	*Samples from original homogenised master-batch before sub-sampling, which were shaken before sample preparation for ICP-OES*
Group 4	5065 -5066 - 5067 - 5068	*Samples from homogenised master-batch before sub-sampling, which were not shaken before sample preparation for ICP-OES*

In order to test the homogeneity, from each sample three independent sub-samples were collected using a 50 µl pipette and diluted with Milli-Q water in a 100 ml volumetric flask. From these diluted solution, an aliquot of 200 µl was used for microwave assisted digestion, using the working programme described in Subsection 4.7.1. After digestion, the extract was diluted in 50 ml volumetric flask with Milli-Q water and analysed by ICP-OES. During the experiment, the NM-300 units have been re-homogenised prior to every sub-sampling, vigorously shaking the bottle for about four minutes.

The following scheme was applied in a four day time:
- *day 1*: first series of microwave assisted digestion of 1-test portion per NM-300 unit;
- *day 2*: second series of microwave assisted digestion of 1-test portion per NM-300 unit and ICP-OES analysis of first series;
- *day 3*: third series of microwave assisted digestion of 1-test portion per NM-300 unit and ICP-OES analysis of second series;

- *day 4*: ICP-OES analysis of third series.

For each day of measurement a new calibration curve was performed. Digested samples were analysed in triplicate and the mean values was used.

In Table 21, silver-content derived from ICP analysis of the three independent sub-samples are reported. It can be noted that mean concentrations of Group 1 and 2, which both come from the normal production process (but different batches including "K") are different from samples drawn under consciously altered conditions. A statistical t-test was applied for the comparison of the two means and the result shows that the means are significantly different at 95% and 99% confidence levels.

Table 21: Concentration of silver in NM-300/NM-300K units derived from ICP-OES analysis for homogeneity study.

	Unit ID	*day 1* conc. (%)	*day 2* conc. (%)	*day 3* conc. (%)	*Mean* conc.(%)	*Std. dev.* (%)
Group 1	0030	10.2	9.9	10.0	9.7	0.4
	0051	10.2	10.2	9.6		
	0021	9.5	9.9	9.1		
	0033	9.7	9.0	9.0		
	0075	9.4	9.5	9.6		
Group 2	0886	8.8	9.2	8.4	8.9	0.3
	0897	8.7	8.8	9.0		
	1048	8.8	8.6	8.7		
	1170	9.4	9.4	9.1		
	1432	9.1	8.7	8.8		
	1468	8.8	9.0	8.6		
	1493	9.2	9.2	9.3		
Group 3	5061	9.0	9.2	9.9	9.4	0.3
	5062	9.6	9.9	9.7		
	5063	9.1	9.5	9.1		
	5064	9.5	9.5	9.0		
Group 4	5065	8.6	8.7	8.6	7.9	0.5
	5066	8.2	8.1	8.0		
	5067	7.8	7.4	7.3		
	5068	7.3	7.5	7.4		

The obtained results were evaluated using 1-way ANOVA. Between-bottle and within-bottle uncertainties (u_{bb} and u_w) were calculated according to the formulas used within ISO 35.

The between-unit (u_{bb}) and within-unit (u_{rep}) standard deviations, which represent relatively the homogeneity uncertainty and the repeatability, were calculated using the following equations:

Equation XXI

$$u_{bb} = \sqrt{\frac{MS_{among} - MS_{within}}{n}}$$

$$u_{rep} = \sqrt{MS_{within}}$$

where MS_{within}, mean squares within the groups, and MS_{among}, mean squares among the groups, were derived from ANOVA; *n* represent the number of test-portions for each unit. Results are reported in Table 22.

Table 22: Results from homogeneity test; ubb and urep being respectively the between unit and within unit standard deviation.

	u_{bb} (%)	u_{rep} (%)
Group 1	3.11	3.12
Group 2	2.25	2.22
Group 3	1.51	3.14
Group 4	7.20	1.65

Observing the results it is evident that only Group 4 is not homogeneous. For this group, samples were drawn from original master-batch containers, which were consciously not re-homogenized before sampling in order to simulate a process-related uncertainty and to further optimize the processes. Starting from this finding, an additional experiment on 5 units of Group 2, where the unit were settled for a week before a new analysis. After a week two test portions were collected from each unit: one at the top/upper part of the dispersion and the other one at the bottom/lower part. Measurements were performed similarly to the experiments described above.

Although it was difficult to sample two-level test portions from the same "depth" of each vial/unit, results (reported in Table 23) show that there is a difference in the silver content between the top/upper and the bottom/lower part of the

respective unit/vial; mean percentage difference between the two levels concentrations is 29%.

Table 23: Results from homogeneity test. Top and bottom portions were taken after settled the units for a week; mixed portions after shaken units for 4 minutes.

Unit ID	upper (%)	lower (%)	Mixed (%)
0886	8.9	11.3	9.2
1048	8.5	10.8	8.9
1432	8.1	12.5	9.2
1468	8.9	13.5	9.3
1493	8.4	12.6	9.7

These findings demonstrate that silver NPs tend to accumulate towards the bottom of the unit or vial over time, together with the stabilising agent; this is also confirmed by the measurements performed for samples obtained from the upper or lower parts of the vials (see Figure 20). Moreover, a T-test was used to compare the mean values from upper and lower parts of the vial, results in rejecting the hypothesis of equal means (95% and 99% confidence level).

Figure 20: Top (right) and bottom (left) diluted test portions.

Finally, units used in this experiment were re-mixed for about four minutes and a test portion from the centre of the solution was taken in order to test if, after shaking, the NM-300 material returns to the original homogenisation level.

Results are shown in the last column of Table 23. Mean difference between the re-mixed unit from this test and from the homogenization one (Table 21) is 4%. Using a t-test to compare the means resulting from the two experiments, the hypothesis of equal means is accepted at a 99% confidence level. This shows that the content of the NM-300 vial can be conditioned by vigorously shaking the vial for four minutes and that a homogenous within-bottle distribution will be reached by this treatment.

In order to test the homogeneity of NM-300K in a dedicated experiment, 20 vials/samples were randomly selected. From each sample three independent sub-samples were collected on three different days. To obtain a concentration more suitable for ICP analysis, about 50 mg of sample were weighted into a volumetric flask and then diluted up to 100 ml with Milli-Q water; weights of representative subsamples are reported in Table 24.

Table 24: Weights of test portions used for dilution.

Sample ID	Day-1 weight (g)	Day-2 weight (g)	Day-3 weight (g)
0078	0.0537	0.0551	0.0564
0079	0.0515	0.0552	0.0557
0082	0.0553	0.0565	0.0550
0085	0.0521	0.0523	0.0556
0095	0.0518	0.0553	0.0559
0103	0.0540	0.0575	0.0556
0110	0.0559	0.0569	0.0559
0117	0.0514	0.0566	0.0552

From these diluted solution, an aliquot of 200 µl was used for microwave assisted digestion, using the working program described in Table 25.

Table 25: Program used for microwave digestion (Vent.=ventilation).

Time	Power
7	250
7	500
5	750
3	Vent.

The following reagents were added to each vessel: 3 ml HCl and 1 ml HNO_3. Also a blank (3 ml HCl + 1 ml HNO_3) was digested. After digestion procedure, vessels were left to cool to ambient temperature and then each extract was filtered in a 50 ml glass flask using a clean glass funnel and a Whatman filter (grade No. 42). The vessel and the vessel cup were rise out for three times with Milli-Q water and the rinse water was filtered in the same flask. At the end, the flask was made up to volume. Samples were stored in a refrigerator at 4ºC.

For ICP analysis, an aliquot of the digested samples was put in the ICP vials. For the calibration curve, a standard blank and five opportunely diluted amounts of silver standard solution were used. During the experiment, the NM-300K units have been re-homogenised prior to every sub-sampling, gently shaking the bottle for about four minutes. The above described working conditions are the same used as described above for testing NM-300/NM-300K homogeneity. For each day of measurement a new calibration curve was performed. Digested samples were analysed in triplicate and the mean values was used.

In **Table 26**, silver-content in the NM-300K bottles, express in %, derived from ICP analysis of the three independent sub-samples is reported; also regression coefficients from daily calibration curves are shown. It is to note that Ag concentration values in each NM-300K bottle were computed using the same density obtained for NM-300 material, equal to *1.10±0.03 kg/l*.

Table 26: Silver concentrations in NM-300K bottles expressed in %.

	Day 1	*Day 2*	*Day 3*
Sample ID	*conc. (%)*	*conc. (%)*	*conc. (%)*
Correl. coeffic.	> 0.9999	> 0.9999	> 0.9999
0078	8.4	9.0	8.8
0079	8.9	9.3	9.1
0082	8.8	9.3	9.4
0085	8.6	8.8	9.1
0095	9.0	9.3	10.5
0103	8.8	10.3	9.6
0110	9.7	9.8	10.3
0117	8.5	8.2	8.8

The obtained results were evaluated using 1-way ANOVA. Between-bottle and within-bottle uncertainties (u_{bb} and u_w) were calculated with the following equations.

Equation XXII

$$u_{bb} = \sqrt{\frac{MS_{among} - MS_{within}}{n}}$$

$$u_w = \sqrt{MS_{within}}$$

where MS_{within}, mean squares within the groups, and MS_{among}, mean squares among the groups, were derived from ANOVA; *n* represent the number of test-portions for each unit. Results for between unit and within unit standard deviation are:

$$u_{bb} = 4.6\ \%$$

$$u_w = 4.9\ \%$$

For NM-300 the mass –related content determined by ICP-OES is 9.7 % with a SD of 0.4 %. For NM-300K the mass-related content is 9.2 % with a SD of 0.6 %. The silver content is statistically not different.

6 Conclusions

The JRC has established the NM representative nanomaterials repository, which hosts today more than 25 different types of NM and a total number of more than 20000 individually labelled samples at its centre in Ispra, Italy. JRC has introduced this series of representative nanomaterials (NM) for testing in support to the Organisation of Economical Cooperation and Development (OECD) - Working Party on Manufactured Nanomaterials (WPMN) as well as European and Member States' projects. These NM are studied already in international scientific co-operations. A number of more than 10000 individual test samples were distributed internationally to National Authorities, research institutions, industrial researchers and scientific stakeholders. The NM-300/NM-300K silver < 20 nm is one of the key materials of the programme, for which the current report presents information on characteristics, stability and homogeneity with special regard to its use and appropriateness for use as representative nanomaterial, performance standard and reference matrix for testing.

Inorganic chemical characterisation of the total silver content and the homogeneity of the Silver NP distribution was performed using photometry and ICP-OES. To this end, a dedicated method was developed and validated according to the principles of ISO 17025. The key properties of size and size distribution were studied in a inter-laboratory comparative study using scanning electron microscopy (SEM) as well as transmission electron microscopy (TEM) and Nanoparticle Tracking Analysis. Results by DLS and Zeta-Sizer Analysis are discussed as well. Furthermore, the leaching of silver ions from the NM-300 was studied after embedding in an acrylic matrix.

The NM-300 silver < 20 nm was characterised a containing silver particles of about 15 nm size with a narrow size distribution with > 99 % number of particles with a size below 20 nm. A second peak of smaller nano-Silver particles of about 5 nm was identified using high resolution TEM. Size and size distribution measurements were reliably performed by use of electron microscopy. Other measurement methods show severe limitations and the results differ

significantly from the electron microscopical results. This could be related to the fact that different measurands are addressed and/or the method performance is limited, e.g. for an NM, which is representative for a real life nanomaterial in use.

For NM-300, the mass-related content determined by ICP-OES is 9.7 % with a SD of 0.4 %. For NM-300K the mass-related content is 9.2 % with a SD of 0.6 %. The silver content is statistically not different. The silver content and particle number of NM-300 was shown to be stable over the time of examination up to 12 months. Silver-ions were released from NM-300 RMN, which was embedded in a poly-acrylic matrix up to a defined concentration level. The ion release from the particles of NM-300 into its matrix under the storage conditions was determined by approximation being below 0.01 w/w%. Ion concentration was found to be stable over time within NM-300 vials under the described conditions. Tests conducted on NM-300 reveal that it is advisable to shake the solution before use, in order to re-homogenise the content of the vial. Deposition can occur during sample storage, but our findings demonstrate that this process is reversible.

With ICP-OES technique, it is possible to detect silver in nano-form after dissolution with an expanded uncertainty of 7%, as demonstrated by the validation study. This is a first step experiment for the determination of total silver content, including the nano-fraction, in complex matrices. Other techniques have to be studied and applied to separate the nano-fraction from the total one.

The properties of NM-300/NM-300K studied and described in this report demonstrate the NM-Series' relevance for use in measurement and testing studies, such as for hazard identification and related to safety of nanomaterials. It serves the need as representative nanomaterial, performance standard and reference matrix for harmonisation and standardisation, method development, optimisation and validation.

APPENDICES

A number of relevant electron micrographs and graphical presentations of analysis of micrographs are collected in the appendices. Due to the fact that instruments and analytical software was used in scientific institutions in a number of European Member States, some text may be displayed in languages other than English. Nevertheless, the context and content is self-explaining. If there may be additional questions, please address them to the corresponding author.

Appendix A

Analysis Procedure for TEM micrographs

Micrographs used for the analysis and corresponding size distribution curves (TEM)
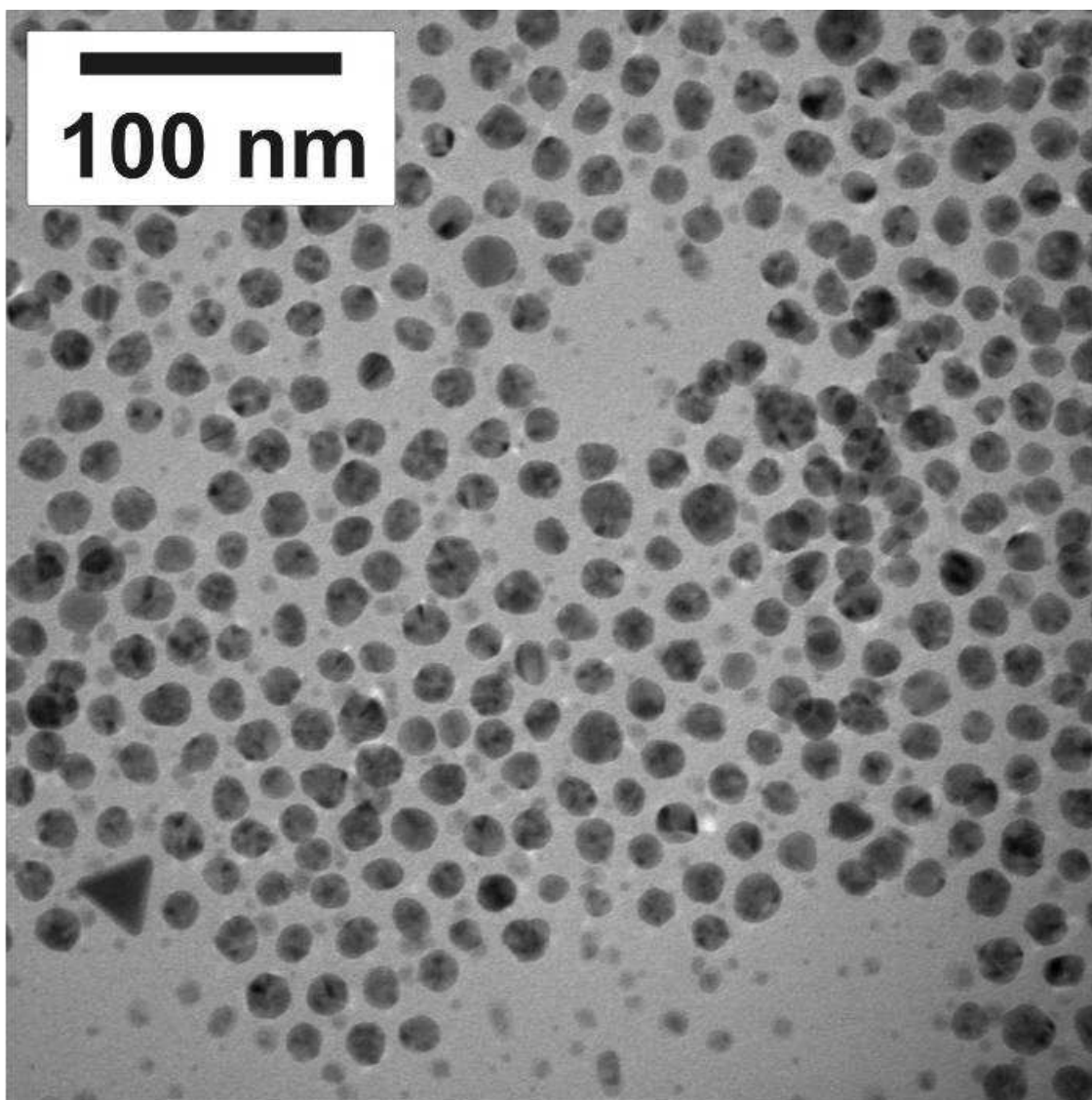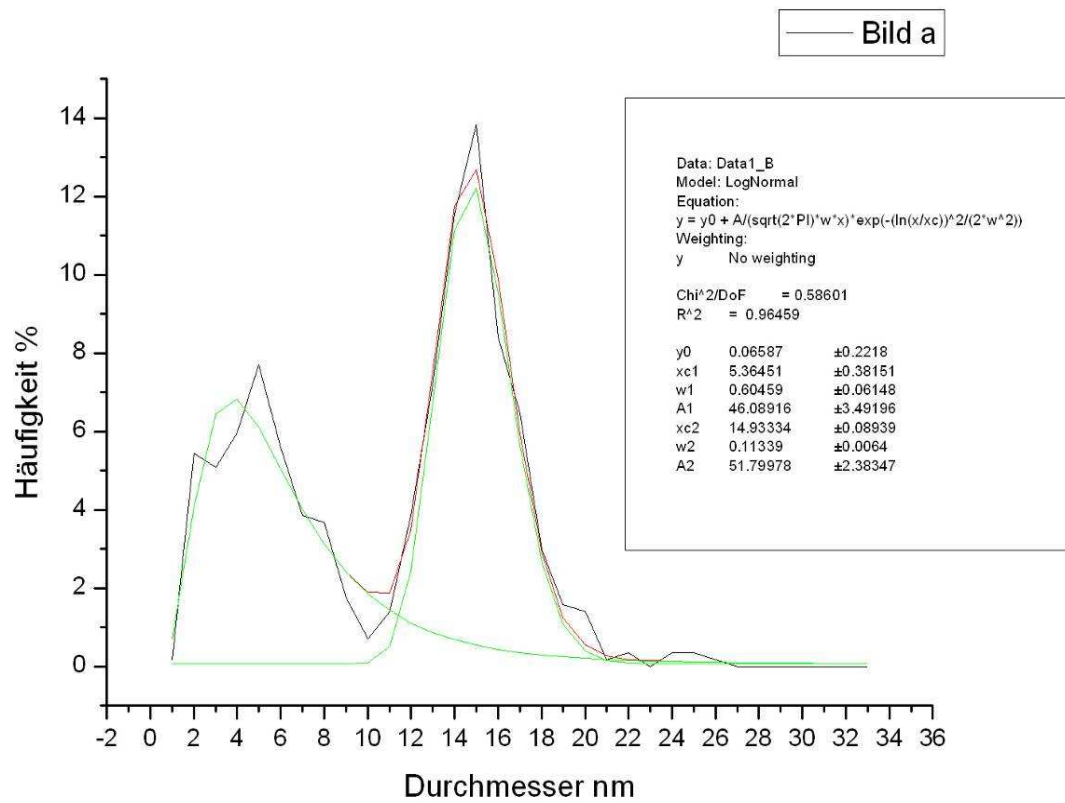
Figure 21: micrograph and corresponding size distribution curves (TEM, Bild a).
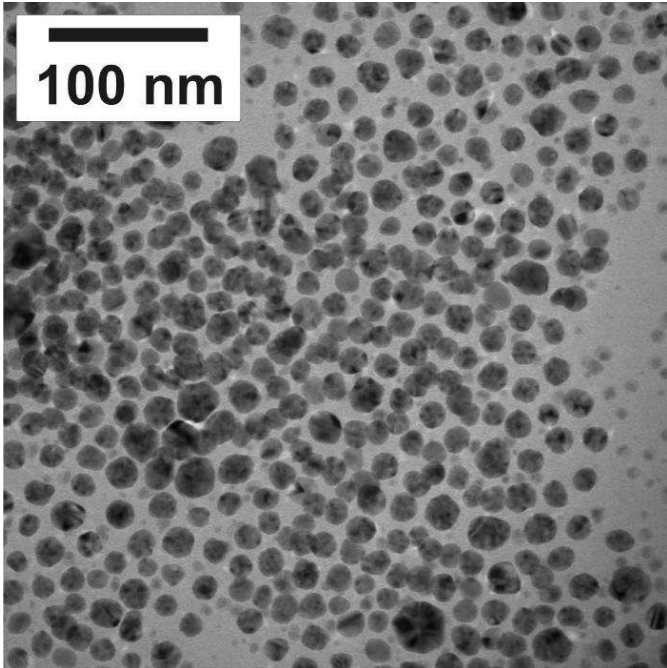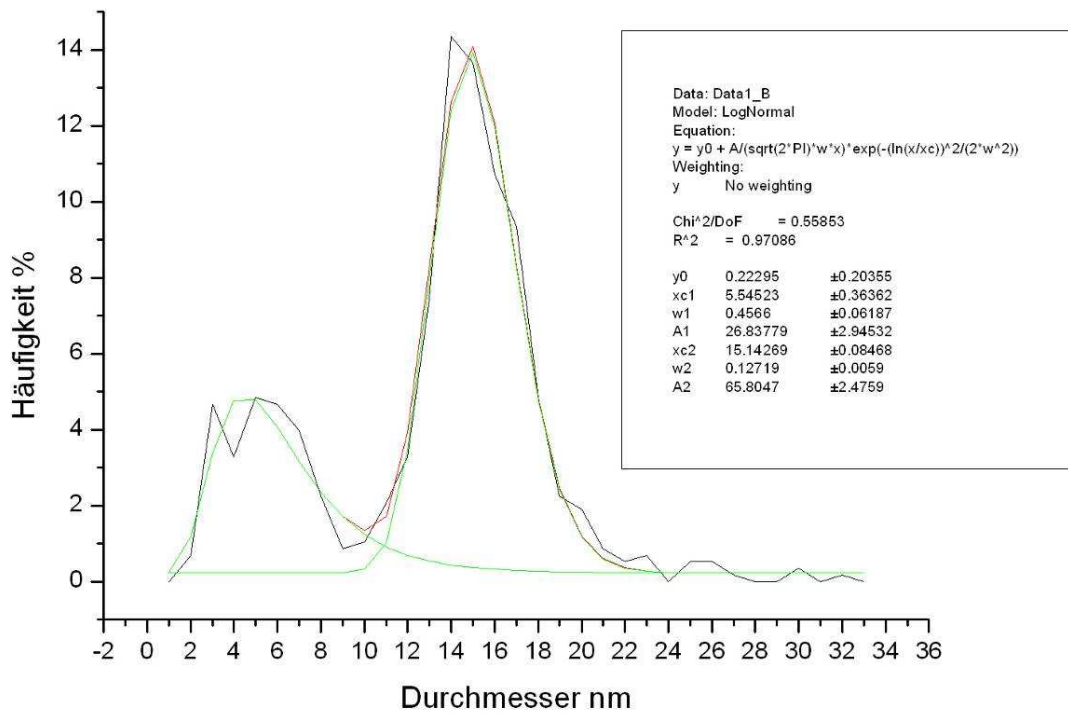
Figure 22: micrograph and corresponding size distribution curves (TEM, Bild b).
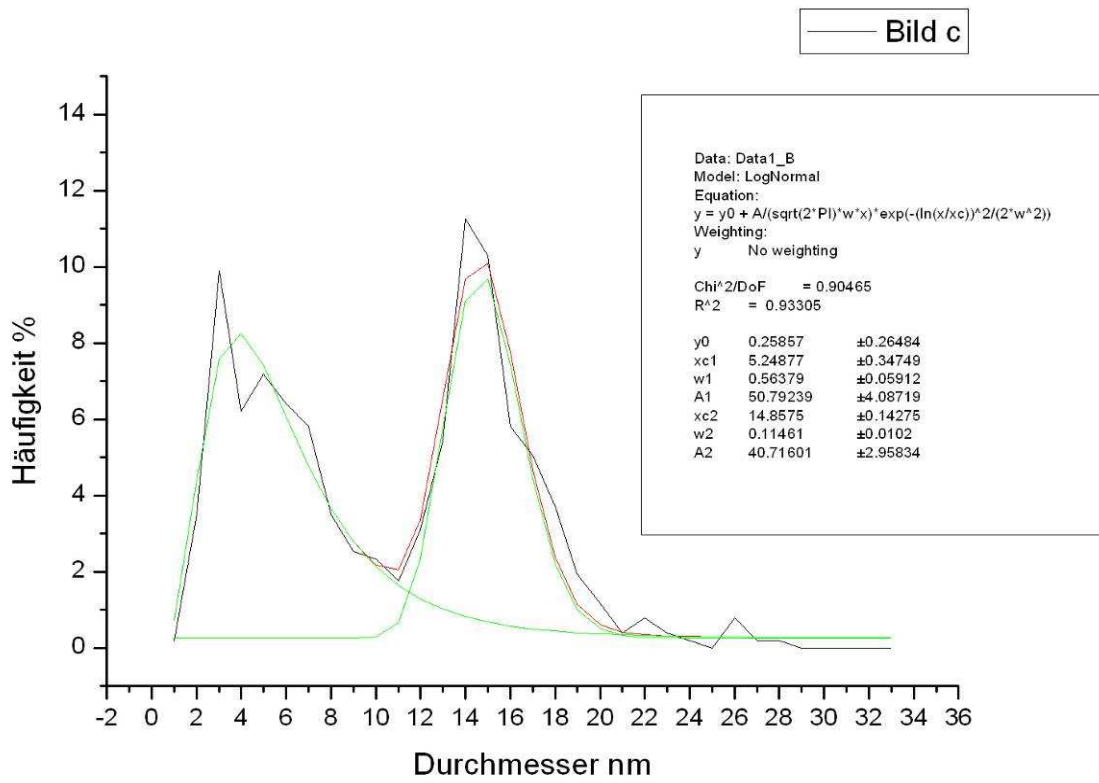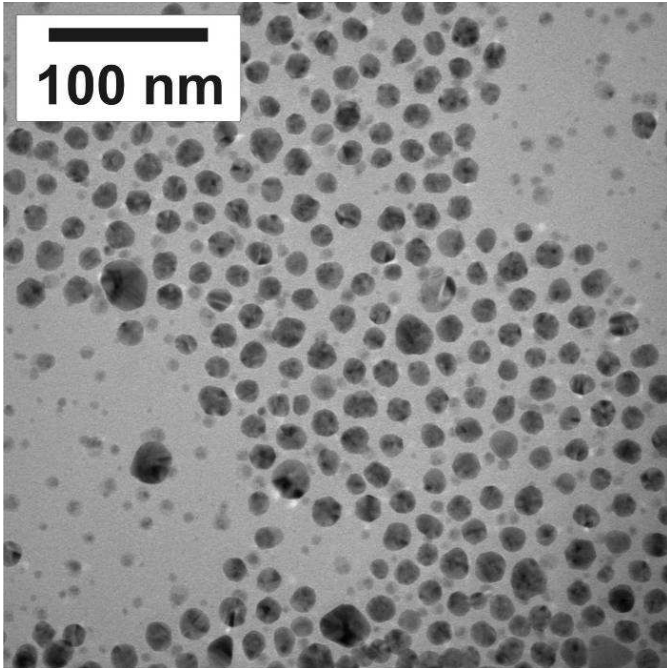
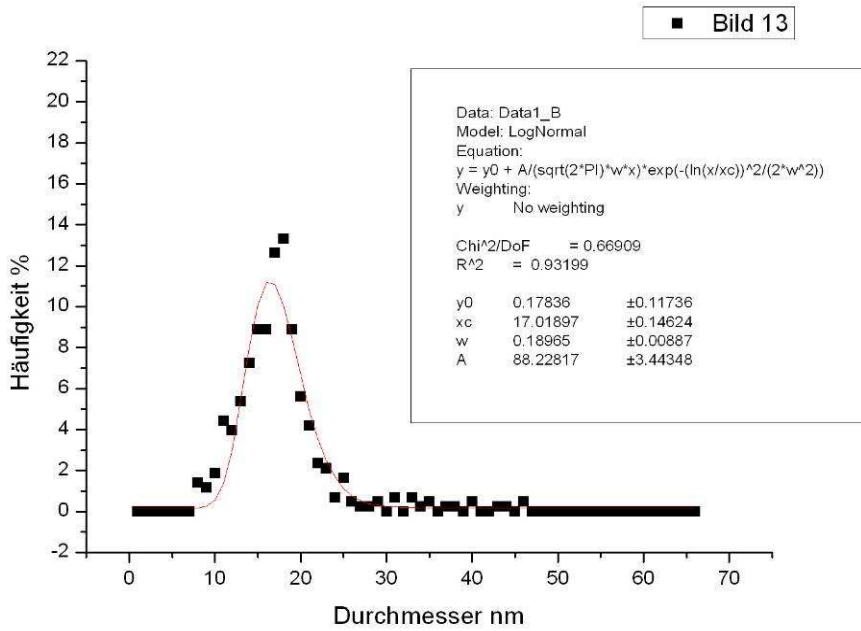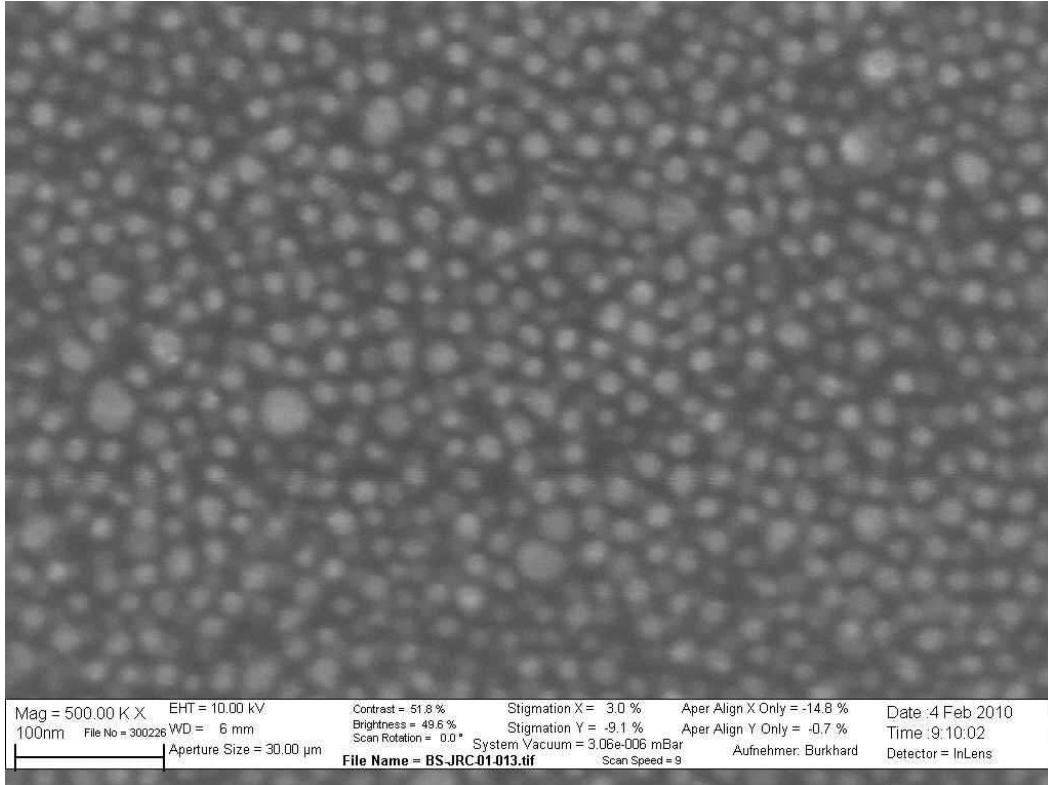Figure 23: micrograph and corresponding size distribution curves (TEM, Bild c).
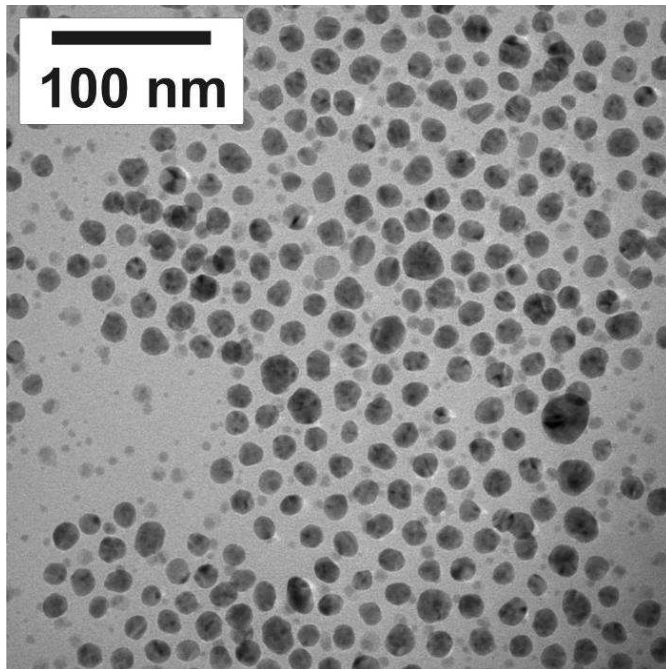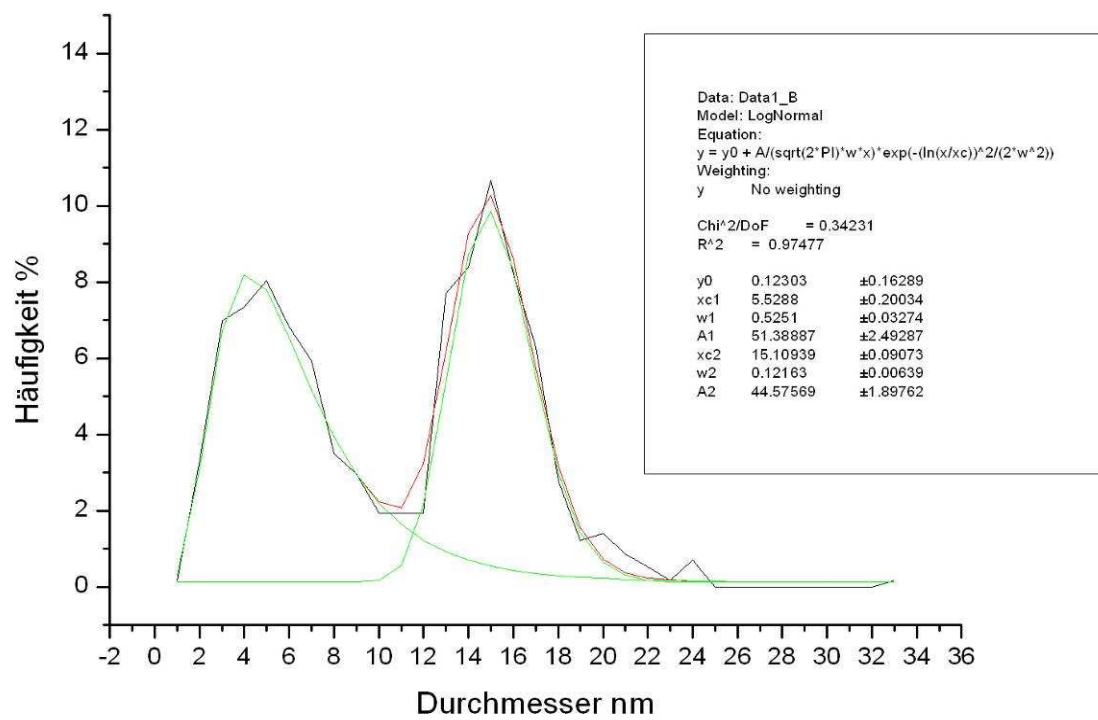
Figure 24: micrograph and corresponding size distribution curves (TEM, Bild d).

Appendix B

Micrographs used for the analysis of silver nanoparticles at TEM by Veterinary and Agrochemical Research Centre.
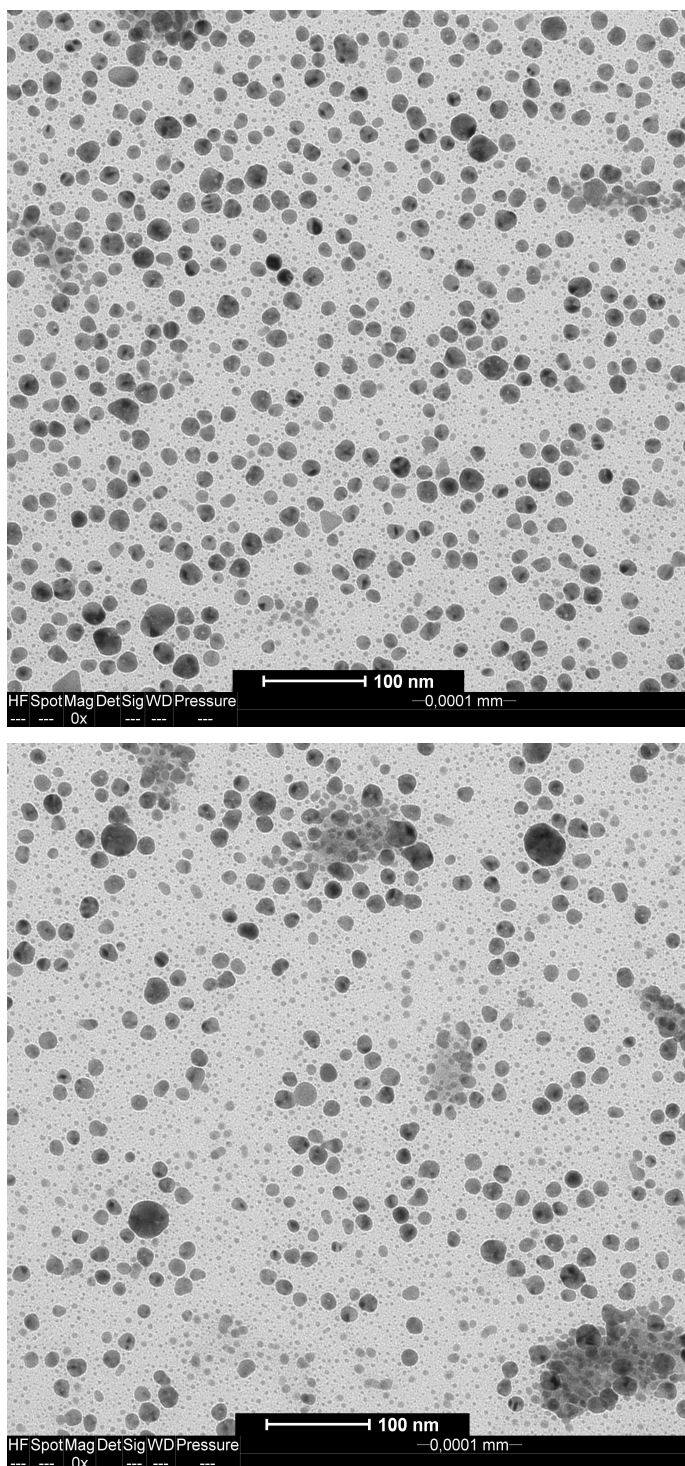
Figure 25: micrographs from TEM for characterization of silver nanoparticles with a mean diameter of 3-4 nm.

Appendix C

1) Analysis Procedure for SEM micrographs

The particles in the SEM micrographs are overlaying each other much less than in the TEM micrographs. Therefore, a semi-automatic procedure with the software SIS Analysis® could be applied to detect and measure individual particles. The different steps of the method are explained based on an example below.

First, the grey level images are calibrated to nanometres. Then the threshold value of the gray level is set in a way that the blurred edges of the particles will get cut off (*cf.* Figure 14).
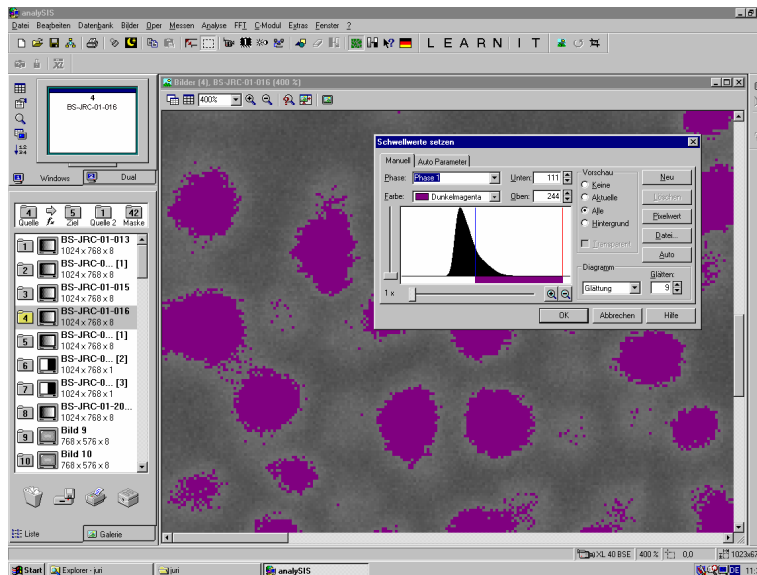
Figure 26: Example of grey level image.

Hereafter, the images are transformed into binary form, so that particles still touching each other can be separated by a morphological filter (Figure 26).
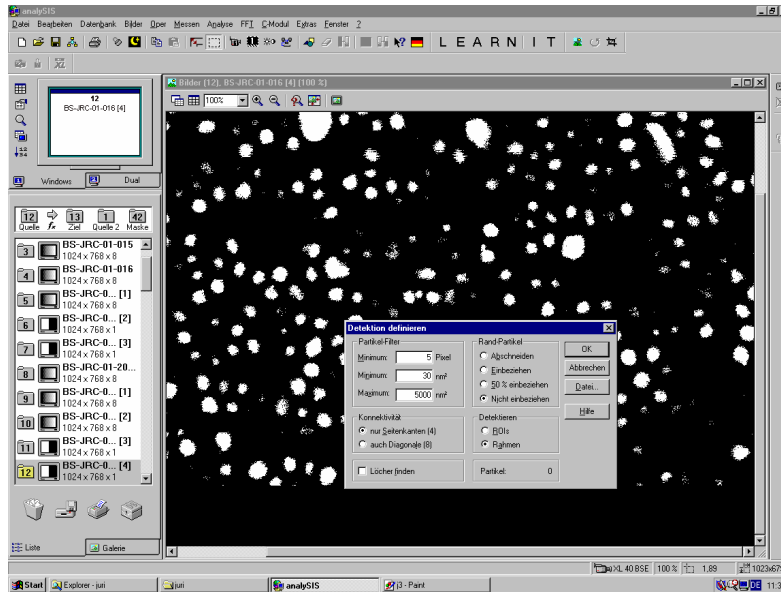
Figure 27: example of binary form image

Now the criteria for particle detection are set, so that particles with an area of less than 30 nm² or more than 5000 nm² are not regarded, which corresponds to particles with diameters less than 6.3 nm or more than 79.9 nm. The lower boundary prevents the detection of false particles created by the fluctuation noise in the images as well as the detection of small particles, which the software can only measure with a high error (the smallest particle detected by the software has actually a diameter of 7.23 nm). Many blurred particles were also ignored in the analysis, as the software could not detect them.

In this way, between 166 and 428 particles were measured in each micrograph. The diameter put out by the software is the average of 24 measurements per particle (each measurement turned by 15°). This should decrease the statistical error of the analysis. However, the arbitrary setting of the threshold value for particle detection creates an error again.

In Figure 27, the original SEM image is compared to the edited image:
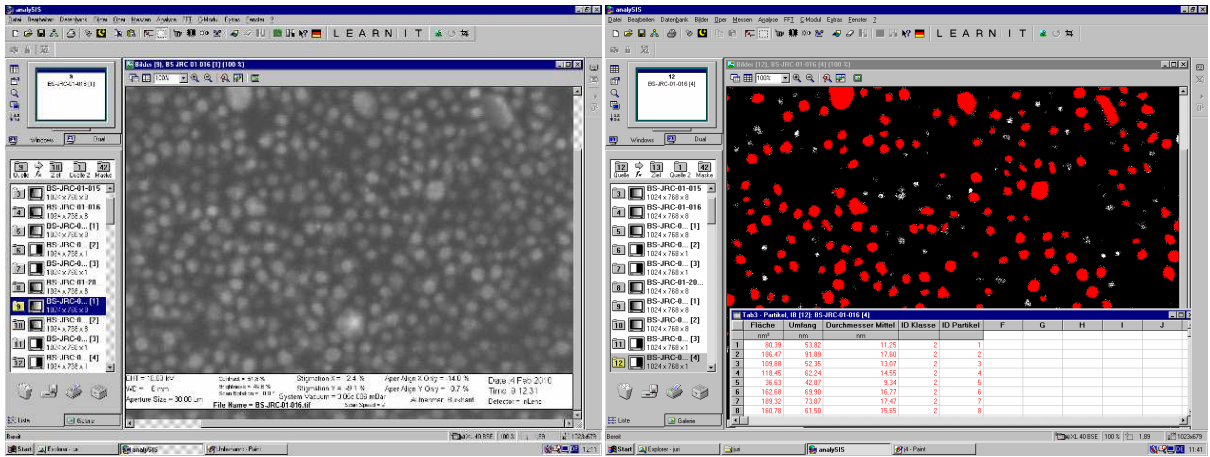
Figure 28: left, original SEM image; right, binary image with detected particles (red) and measured diameters in a table.

Micrographs and size distribution curves (SEM)

Micrographs used for the analysis and corresponding size distribution curves (SEM)

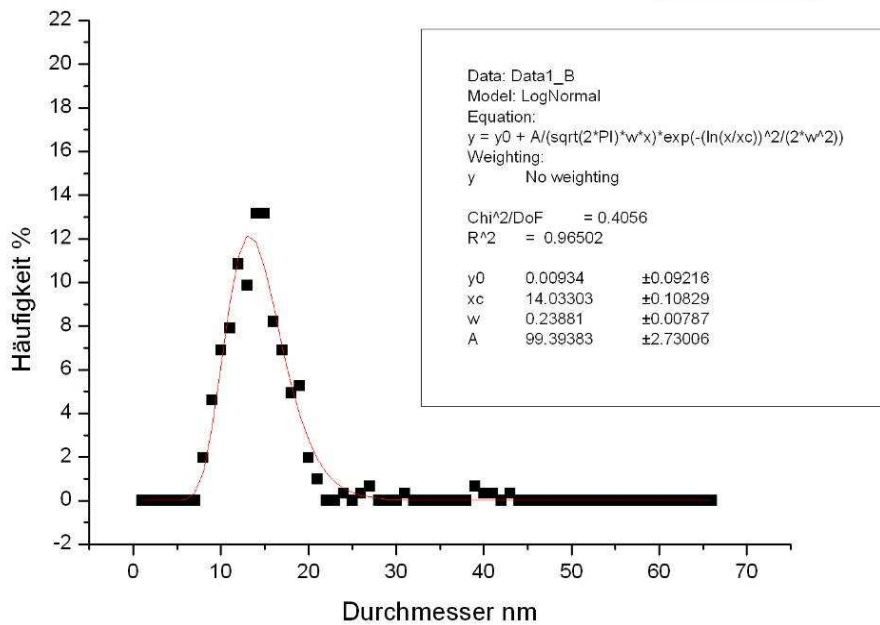**Figure 29: micrograph and corresponding size distribution curve (SEM, Bild 13).**
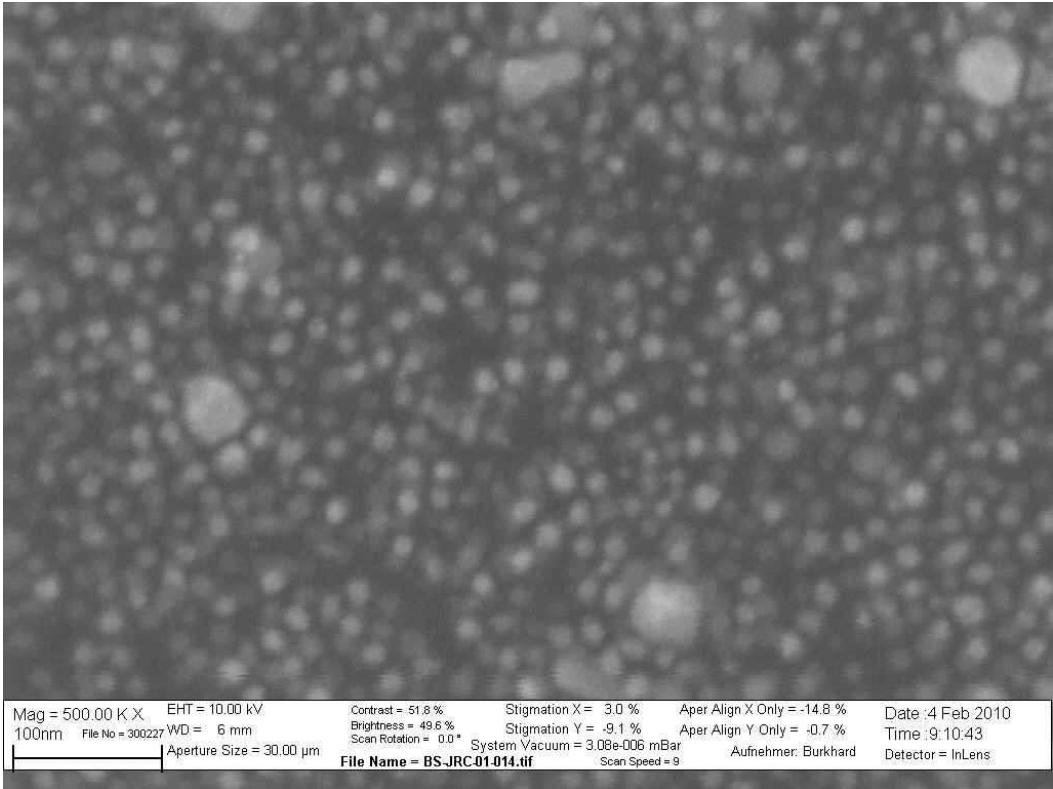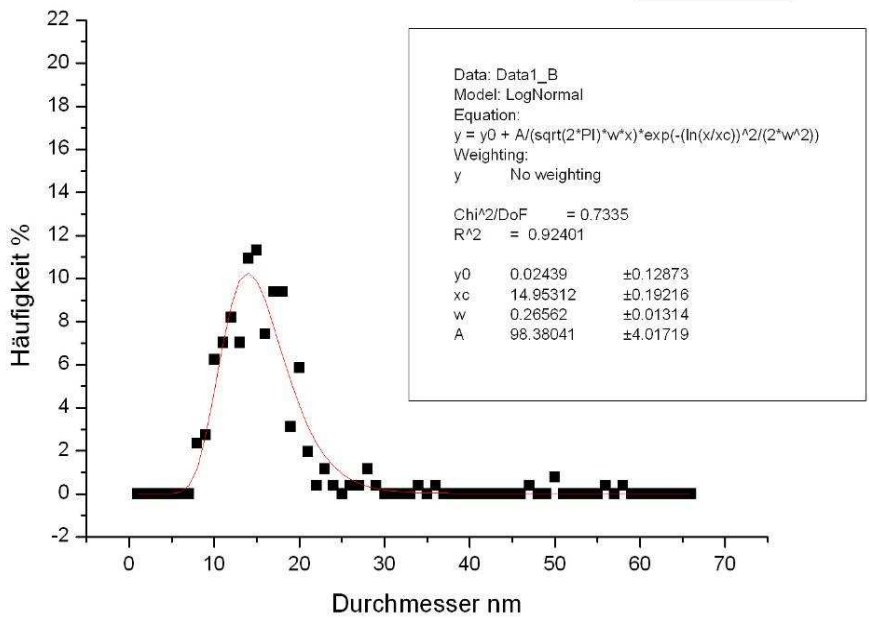
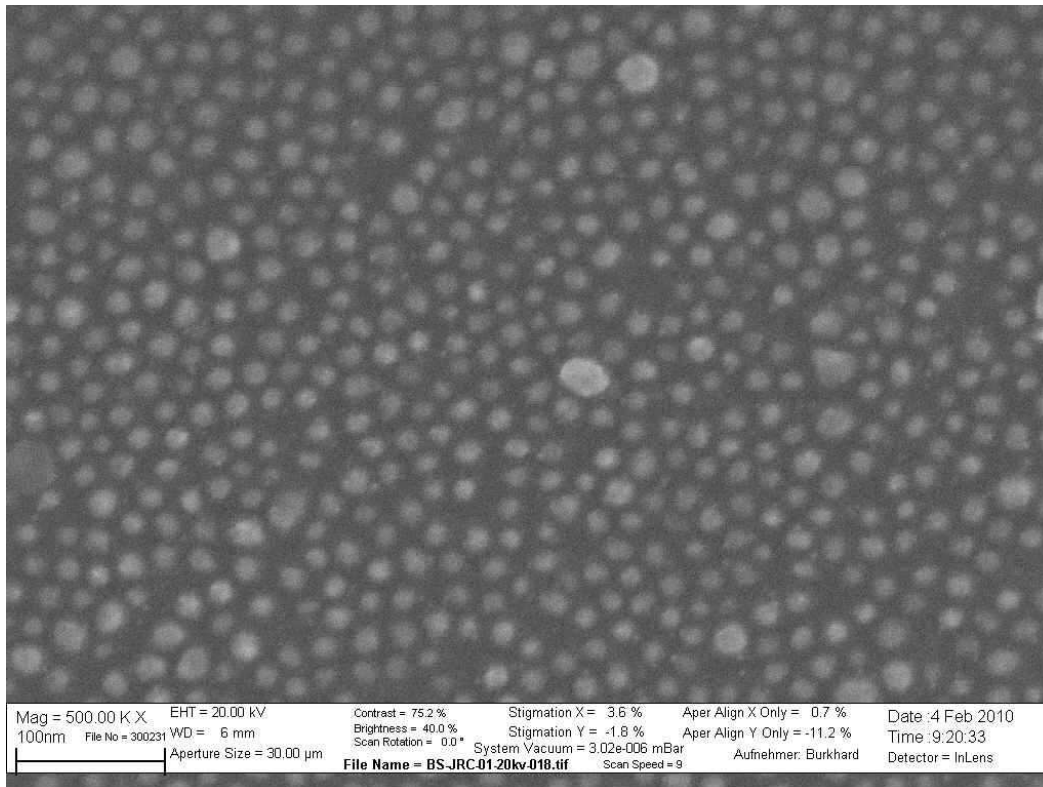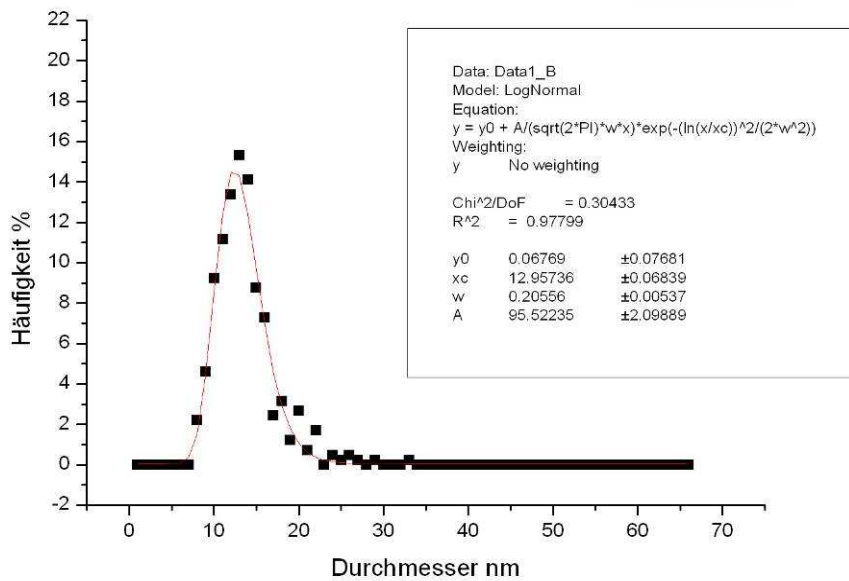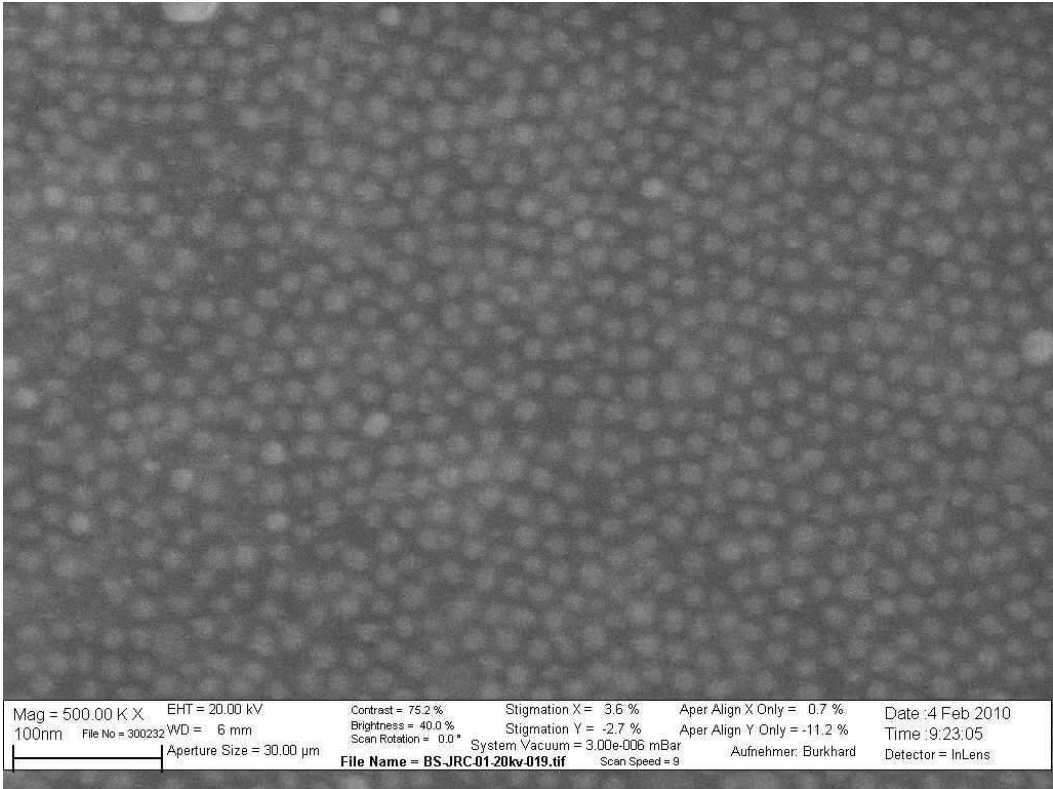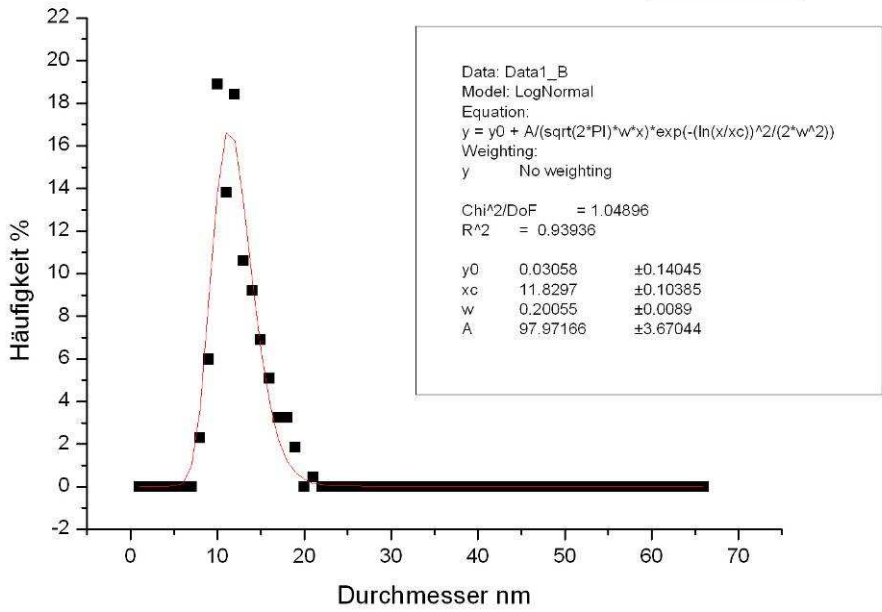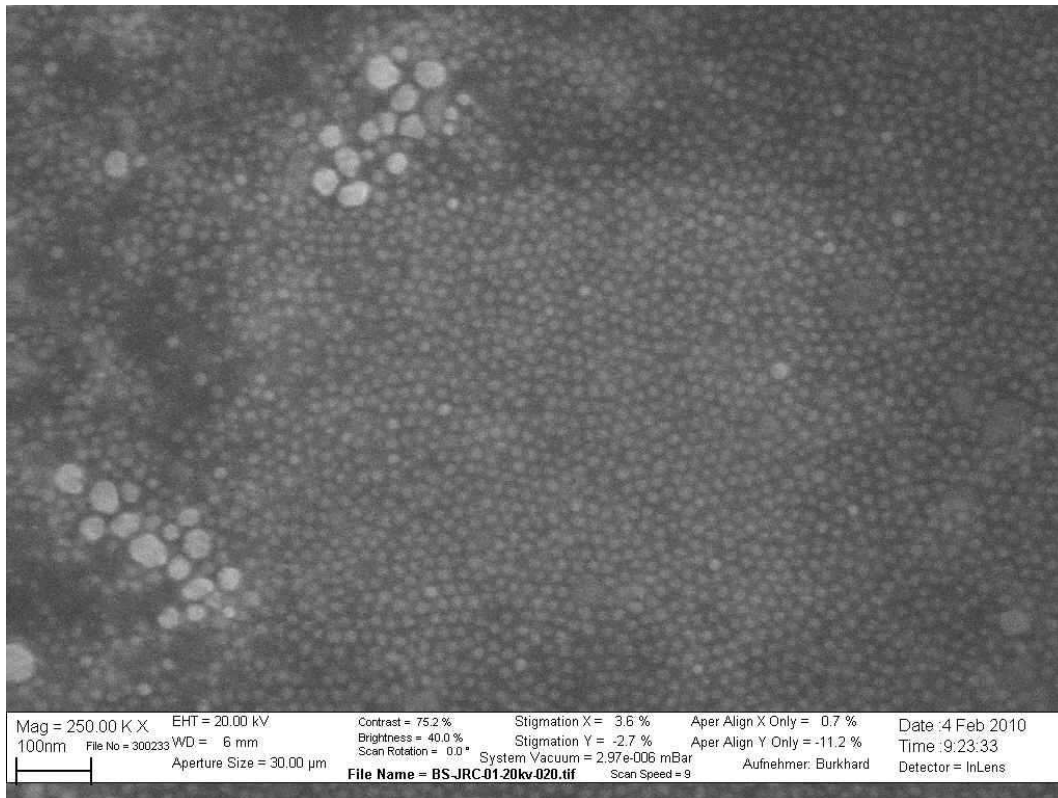Figure 30: micrograph and size distribution curve (SEM, Bild 14).
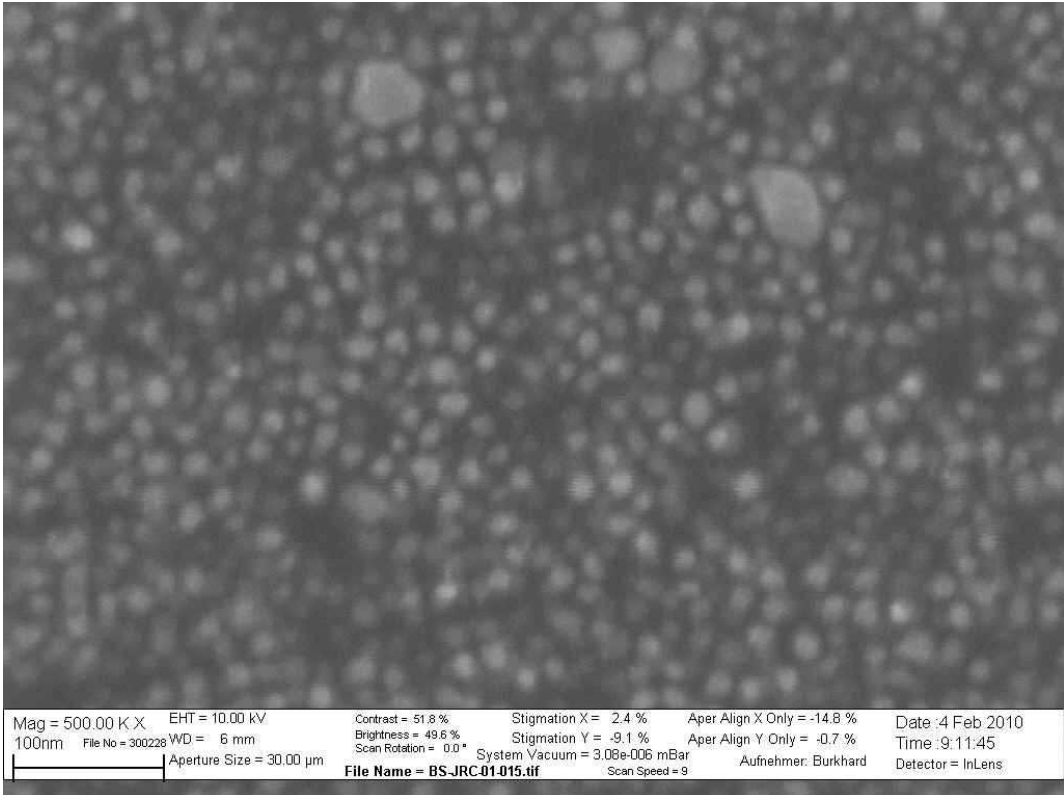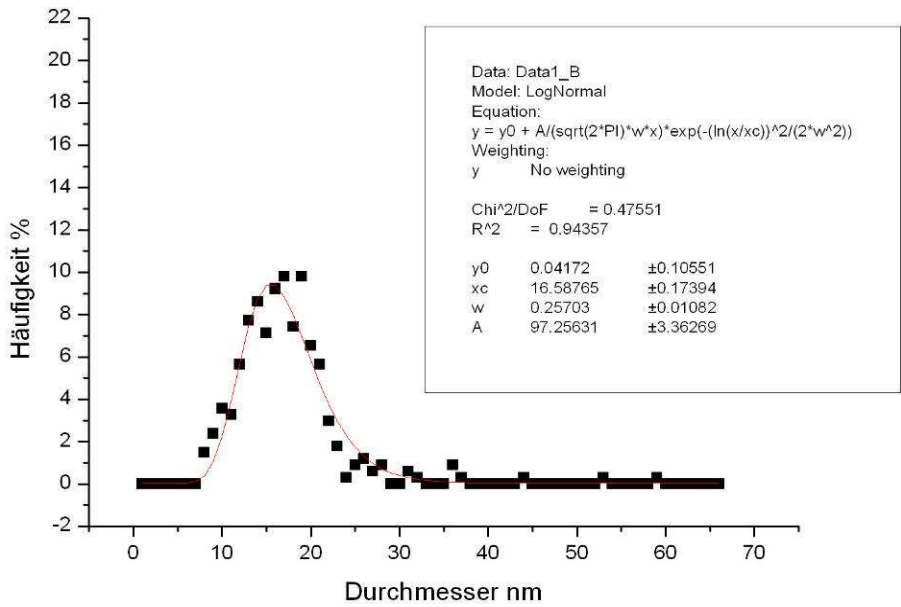
Figure 31: micrograph and size distribution curve (SEM, Bild 15).
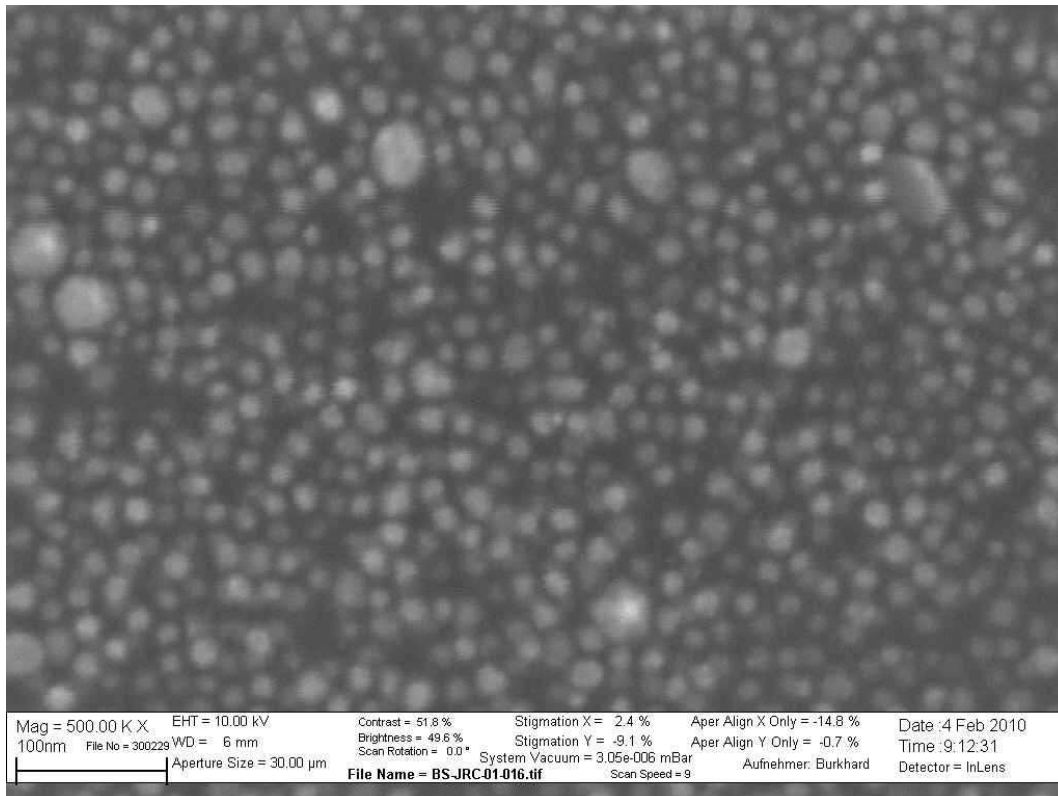
Figure 32: micrograph and size distribution curve (SEM, Bild 16).

Figure 33: micrograph and size distribution curve (SEM, Bild 18).

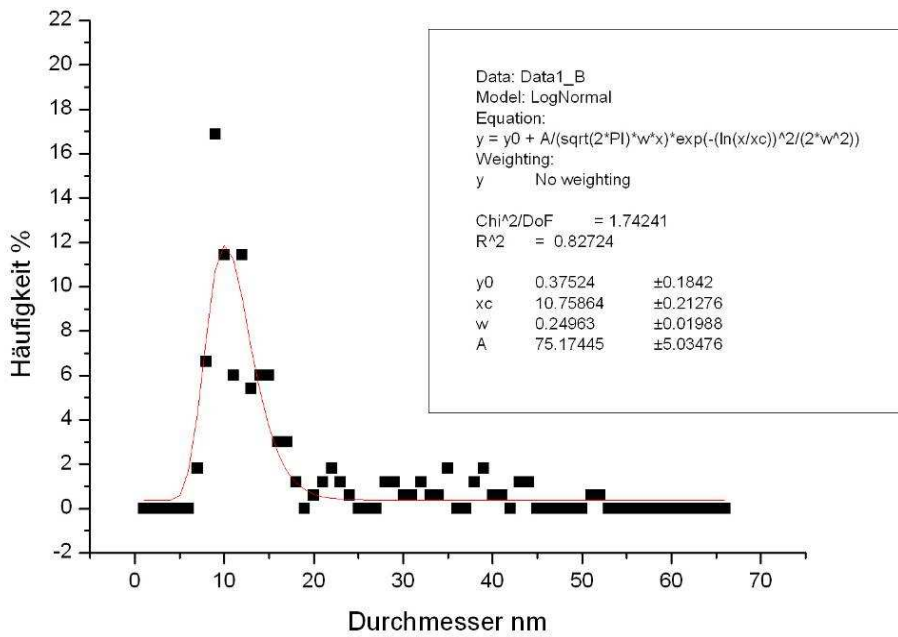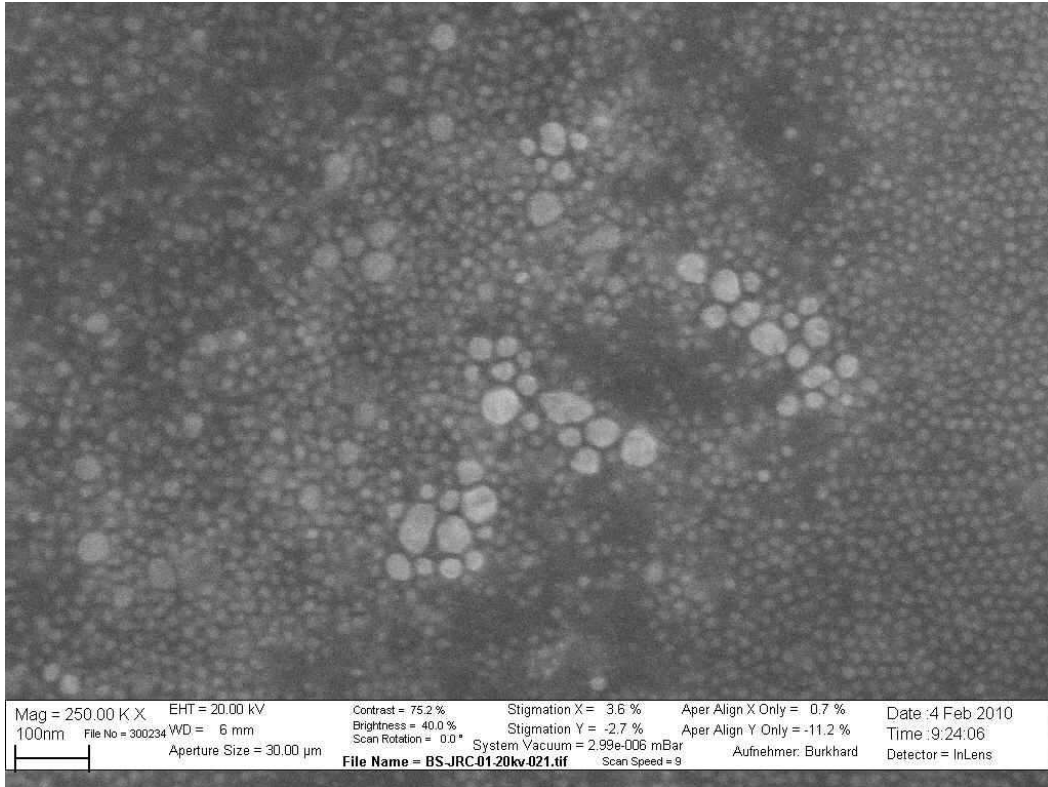**Figure 34: micrograph and size distribution curve (SEM, Bild 19).**

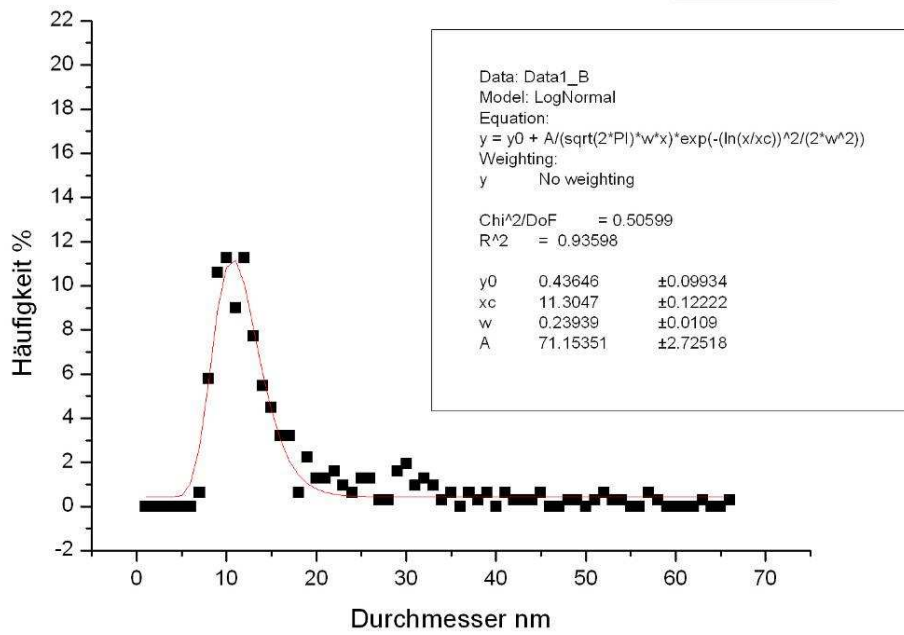Figure 35: micrograph and size distribution curve (SEM, Bild 20).

Figure 36: micrograph and size distribution curve (SEM, Bild 21).

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Abstract

The European Commission Joint Research Centre (JRC) provides scientific support to European Union policy regarding nanotechnology and public health in a sustainable environment. Over the last three years, the JRC has focused part of its work on establishing and applying a priority list (NM-Series) of Representative Manufactured Nanomaterials (RMNs) in support of one of the most comprehensive nanomaterial research programmes that is currently being carried out: the Organisation for Economic Co-operation and Development's (OECD) Working Party on Manufactured Nanomaterials (WPMN) Sponsorship Programme. The JRC's provision of NM-Series RMNs to the OECD WPMN Sponsorship Programme ultimately enables the development and collection of data on characterisation, measurement, toxicological and eco-toxicological testing, and risk assessment or safety evaluation of nanomaterials. Representative nanomaterials are of utmost importance to be made available to the international scientific community to enable innovation and development of safe materials and products.

The present report describes the characterisation of NM-300, a RMN nano-Silver dispersion containing nanoparticles (NPs) of < 20 nm, originating from a single batch of manufactured nano-Silver, used for measurement and testing for hazard identification, risk and exposure assessment studies. The first series of sub-samples created from the batch is labelled NM-300, while the further processed series of NM 300 is labelled with an additional "K" as NM 300K, in order to signify a continued processed number of sub-samples from the same batch of raw material. This NM-Series material was produced in the frame of the JRC programme on nanomaterials. It was studied by a number of international laboratories including the JRC IES analytical laboratory. Inorganic chemical characterisation of the total silver content and the homogeneity of the silver distribution were performed using photometry and ICP-OES. To this end, a dedicated method was developed and validated according to the principles of ISO 17025. Key properties of size and size distribution were studied in an inter-laboratory comparative study using SEM as well as TEM and Nanoparticle Tracking Analysis. Furthermore, the release of silver ions from the NM-300 was studied after embedding in an acrylic matrix.

The NM-300 silver <20 nm was found to contain silver particles of about 15 nm size with a narrow size distribution of 99 % of the particle number concentration exhibiting a diameter of below 20 nm. A second, much smaller abundance of particles was identified by TEM to have narrow diameter distribution of around 5 nm. The silver content and particle number of NM-300 was shown to be stable over the time of examination, lasting up to 12 months. Silver-ions were released from NM-300 silver nanomaterial, which was embedded in a poly-acrylic matrix up to a defined concentration level.

The properties of NM-300 studied and described in this report demonstrate its relevance for use in measurement and testing studies, such as for hazard identification, related to the safety of nanomaterials. The studies were performed in close collaboration with the Fraunhofer Institute for Molecular and Applied Ecology (Fh-IME), German Institute of Energy and Environmental Technology e.V. (IUTA), the Swiss Federal Laboratories for Materials Science and Technology (EMPA), RAS Material Science GmbH, Germany, and the Veterinary and Agrochemical Research Centre (VAR) in Belgium.

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