Detection and fate of engineered nanoparticles in aquatic systems

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Abstract

The proliferation of nanotechnology has prompted discussions over the safety of these materials to human health and the environment as their environmental fate and impact is widely unknown. This is partly due to the lack of suitable analytical techniques to detect and characterise engineered nanoparticles in the environment.

This thesis aims to provide a better understanding of the environmental fate of engineered nanoparticles by developing analytical methods suitable for nanoparticle analysis in aquatic systems and employing these to laboratory-based environmental fate studies.

As a first step the applicability of existing analytical techniques to nanoparticle detection and characterisation in complex media were critically reviewed and potentially suitable approaches were identified.

A comparison of submicron microscopic techniques revealed that the use of the novel WetSEM™ approach is a promising complimentary tool for the visualisation of nanoparticles in aquatic systems avoiding drying artefacts *e.g.* salt crystals compared to conventional submicron microscopic techniques.

Hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry was developed using gold nanoparticles as sizing standards for the size and elemental characterisation of nanoparticles in aquatic systems. The method is fast, robust, features a high sample throughput and fractionates particles over a wide size range (5 – 300 nm). The method was validated by electron microscopy.

This technique was then applied to assess the fate of silver nanoparticles in activated sewage sludge processes. It was found that the majority of the silver partitioned to the solid sludge residue. However, a fraction of the silver nanoparticles can survive wastewater treatment in the nanoform and therefore might be released to rivers and streams. A modelling approach based on the obtained data was developed to predict nanosilver concentrations in the surface waters.

Finally, based on experimental work and published literature, approaches for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles are critically discussed.

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Glossary

AEM = Analytical electron microscopy

AES = Auger electron spectroscopy

AFM = Atomic force microscopy

ATOFMS = Aerosol time of flight mass spectrometry

BET = Brunauer Emmett Teller

C60 = Fullerenes, based on C60 molecules

C70 = Fullerens, based on C70 molecules

CE = Capillary electrophoresis

CFUF = Cross flow ultrafiltration

CPC = Condensation particle counter

Cryo-TEM = Cryo transmission electron microscopy

DLS = Dynamic light scattering

DMA = Differential mobility analyser

EEM = 3D fluorescence excitation-emission matrix

EM = Electron microscopy

EM = Electrophoretic mobility

ENPs = Engineered nanoparticles

ESEM = Environmental scanning electron microscopy

FCS = Fluorescence correlation spectroscopy

FFF = Field flow fractionation

HDC = Hydrodynamic chromatography

HPLC = High performance liquid chromatography

ICP-MS = Inductively coupled plasma mass spectrometry

LIBD = Laser induced break down diffraction

LOD = Limit of detection

MLSS = Mixed liquor suspended solids

NMR = Nuclear magnetic resonance spectroscopy

NOM = Natural organic matter

NPs = Nanoparticles

NSOM = Near-field scanning optical microscopy

PCS = Photon correlation spectroscopy

PEC = Predicted environmental concentration

PNEC = Predicted no effect concentration

QELS = Quasi elastic light scattering

Raman = Raman spectroscopy

rRT = Relative retention time

SANS = Small angle neutron scattering

SEC = Size exclusion chromatography

SEM = scanning electron microscopy

SIMS = Secondary ion mass spectrometry

SLS = Static light scattering

SMPS = Scanning mobility particle sizer

SPMS = Single particle mass spectrometer

STEM = Scanning transmission electron microscopy

STXM = Scanning transmission X-ray microscopy

TEM = Transmission electron microscopy

TGA = Thermo-gravimetric analysis

TLD = Through-the-lens detector

TOF-MS = Time-of-flight mass spectrometry

UV/Vis = UV/Vis spectroscopy

WetSEM = Wet scanning electron microscopy

WetSTEM = Wet scanning transmission electron microscopy

XAS = X -ray absorption spectroscopy

XPS = X-ray photoelectron spectroscopy

XRD = X-ray diffraction

XRF = X-ray fluorescence spectroscopy

XRM = X-ray microscopy

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Author's declaration

Chapters 2 to 6 and appendices have been written as papers for international peer-reviewed journals. The current publication status of the papers is presented in table 0.1. All these papers have been reworked, so that they are presented in a consistent style and format in this thesis. For those papers, which have been published, copyright rests with the publishers.

The papers (chapters 2 to 6) have been written by the candidate as leading author. However, it should be noted that the papers have gained in quality through suggestions, advice and editing from the co-authors. Chapters 2 & 6, which are published papers, have also benefited from the comments of the anonymous referees as part of the review process. The candidate has co-authorship on the papers presented in the appendices.

The work in this thesis was undertaken as a PhD student in the EcoChemistry Team at the Central Science Laboratory in York and the University of York (November 2005 – November 2008). Parts of the experiment for chapter 5 were undertaken at SEAC Unilever in Colworth. The candidate was funded by Unilever, UK.

Table 0.1. Status of the papers presented in this thesis with respect to the publication process.

Chapter	*Authors*	*Title*	*Status*	*Journal*
2	Tiede K, Boxall ABA, Tear SP, David H, Lewis J, and Hassellöv M	Detection and characterisation of engineered nanoparticles in food and the environment	Published in 2008	*Food Additives & Contaminants* 25(7): 795-821
3	Tiede K, Tear SP, David H, and Boxall ABA	Imaging of engineered nanoparticles under fully liquid conditions in environmental matrices	Submitted	*Water Research*
4	Tiede K, Boxall ABA, Tiede D, Tear SP, David H, and Lewis J	Size characterisation of inorganic nanoparticles in 'real' environmental samples using hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry	Submitted	*Journal of Analytical Atomic Spectroscopy*
5	Tiede K, Wang X, Gore D, Boxall ABA, David H, Tiede D, Baxter M, Tear SP, and Lewis J	Fate of engineered silver nanoparticles in activated sludge systems	Submitted	*Environmental Science & Technology*
6	Tiede K, Hassellöv M, Breitbarth E, Chaudhry Q, and Boxall ABA	Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles	In press	*Journal of Chromatography A*
Appendix 1	Boxall ABA, Tiede K, Chaudhry, Q	Engineered nanomaterials in soils and waters: how do they behave and do they pose a risk to human health?	Published in 2007	*Nanomedicine 2(6): 919-927*
Appendix 2	Hassellöv M, Readman JW, Ranville JF, Tiede K	Nanoparticle analysis and characterisation methodologies in environmental risk assessment of engineered nanoparticles	Published in 2008	*Ecotoxicology 17: 344-361*

Chapter 1

Introduction

Nanomaterials are commonly regarded as materials with at least one dimension below 100 nm (Borm and Muller-Schulte 2006), although there is no official definition. They include nanofilms and coatings (< 100 nm in one dimension), nanotubes and wires (< 100 nm in two dimensions) and nanoparticles (< 100 nm in three dimensions) (Hochella 2002). Their chemical variety covers metals, metal oxides, polymers, carbons, biomolecules and clays in many different shapes and forms (Tran *et al.* 2005). Nanoparticles can occur naturally (*e.g.* in ashes, as soil particles or biomolecules), be produced unintentionally (*e.g.* in diesel exhaust) or be intentionally engineered (Banfield and Zhang 2001; Sharpe 2006). This review will mainly focus on engineered or manufactured nanoparticles (ENPs).

As a consequence of their size, nanoparticles show different physico-chemical properties compared to their respective bulk material. These include changes in optical properties, which can cause changes in colour (*e.g.* gold colloids appear as deep red), thermal behaviour, material strength, solubility, conductivity and (photo) catalytic activity (Hochella 2002; Kamat 2002; Burleson *et al.* 2004). Nanoparticles are effectively a bridge between atomic or molecular structures and bulk materials (Henglein 1993). For example, nanoparticles made of semi-conducting materials and with a size between ~ 1 and 10 nm (corresponding to the diameter of ~ 10 to 50 atoms) are small enough to show quantum effects (quantization of electronic energy levels) and are typically called quantum dots (Rao *et al.* 2002). Probably the most significant influence on the behaviour of nanoparticles, however, is the change in surface-to-volume ratio (Banfield and Zhang 2001). Volume decreases with size but the proportion of atoms at the particle surface increases, and, therefore, the surface

properties can dominate the properties of the bulk material (Waychunas 2001). Furthermore, the structure and properties of the surfaces of nanoparticles are substantially modified compared to the surfaces of the same materials in bulk form owing to the proportionally high curvature of the nanoparticle surfaces, more surface defects and edges, as well as the presence of highly catalytically active sites (Madden and Hochella 2005). Additionally, targeted change in surface properties of ENPs can be achieved by coating or functionalisation of nanoparticles.

The potential benefits of engineered nanomaterials have been long recognised but not until recently has the step from research to manufacture and use been made. Engineered nanomaterials are now being manufactured in ever increasing quantities and finding application in a wide range of products and sectors, including medicines, cosmetics, clothing, engineering, electronics and environmental protection (Ponder *et al.* 2001; Obare and Meyer 2004; Aitken *et al.* 2006; Chaudhry *et al.* 2008). Current applications range from antibacterial wound dressings and clothing to reinforced tennis rackets to advanced, transparent sun protection.

In the food sector, the uses of nanotechnology derived food ingredients, additives, supplements and contact materials are expected to grow rapidly. Chaudhry *et al.* (2008) claim that, worldwide, over 200 companies are conducting R&D into the use of nanotechnology in either agriculture, engineering, processing, packaging or delivery of food and nutritional supplements. Food safety will also potentially benefit with the introduction of nano-based detectors, sensors and labelling (Weiss *et al.* 2006). In some countries, nanomaterials are already used in food supplements and food packaging, with nanoclays as diffusion barriers and nano-silver as antimicrobial agents (Sanguansri and Augustin 2006; Chaudhry *et al.* 2008; Corporate watch 2007; table 1.1).

Table 1.1. Examples for applications of nanomaterials in consumer products.

Application	*Nanotype*	*Reference*
Imperm® food & beverage packaging by Nanocor®	Nanoclay composite	Chaudhry *et al.* 2008
Novasol® food supplement by Aquanova®	Soy isoflavones	Chaudhry *et al.* 2008
Nanotea® nano delivery system by Become Industry & Trade Co. Ltd.	Selenium	Chaudhry *et al.* 2008
Boots® Soltan® facial sun defence cream – containing Optisol® by Oxonica® Ltd	Manganese-doped TiO_2	Corporate watch 2007 (Internet)
Leorex® skin care cosmetics by GlobalMed®	Silica	Corporate watch 2007 (Internet)
Fullerene C60 day & night cream by Zelens®	Fullerene C60	Corporate watch 2007 (Internet)
Envirox™ fuel borne catalyst by Oxonica® Ltd	Cerium oxide	Corporate watch 2007 (Internet)
Acticoat® wound dressings by Smith & Nephew	Silver	Corporate watch 2007 (Internet)
NanoCluster™ delivery system for food products by RBC Life Sciences Inc.®/USA	Nanopowder of unknown composition	Chaudhry *et al.* 2008
Aegis® OX oxygen scavenging barrier resin for PET bottles by Honeywell	Polymerised nanocomposite	Chaudhry *et al.* 2008
Various clothing lines by Brooks Brothers, manufacturer Nanotex	Nano fibre	Corporate watch 2007 (Internet)
Various washing machines by Samsung, manufacturer Nanogist	Silver	Corporate watch 2007 (Internet)
Various refrigerators by Daewoo, manufacturer Nanogist	Silver	Corporate watch 2007 (Internet)

The proliferation of nanotechnology has prompted discussions over the safety of these materials to human health and the environment. It is almost inevitable that humans will be exposed to engineered nanoparticles; for example, due to migration of nanoparticles from food packaging into food, as well as from the application of creams directly to the skin. In addition, unintended (*e.g.* waste, wastewater, sludge) and intended (*e.g.* groundwater remediation) release of nanoparticles into the environment may lead to indirect human exposure (*e.g.* via drinking water, food chain, etc.).

The pulmonary toxicity of airborne particles (mostly referred to as ultrafine particles < 10 μm) has been well studied and it is known that toxicity is strongly related to particle size (Brown *et al.* 2001; Hasegawa *et al.* 2004; Geiser *et al.* 2005; Frampton *et al.* 2006). However, the toxicity of engineered nanoparticles and their effects on human health, as well as their environmental fate and impact in water and soil, is still widely unknown (Burleson *et al.* 2004), although some studies suggest (eco-) toxicity. It has been reported that different types of nanoparticles can cause cytotoxicity and cross-cellular layers (Shiohara *et al.* 2004; Koch *et al.* 2005; Chen and von Mikecz 2005; Hardman 2006; Brunner *et al.* 2006), as well as accumulate in tissue (Bullard-Dillard *et al.* 1996) and cause increased production of oxyradicals (Li *et al.* 2003). Toxicity of fullerenes and TiO_2 nanoparticles to *Daphnia*, large mouth bass, zebra fish and other aquatic species has also been reported (Oberdorster 2004; Oberdorster *et al.* 2006; Lovern and Klaper 2006; Yeo and Kang 2008). Studies have also looked at impacts on terrestrial systems, for example alumina nanoparticles have been shown to be phytotoxic (Yang and Watts 2005). Fullerenes, silver and other nanoparticles have also shown antibacterial behaviour, *e.g.* in healthcare applications and in aquatic environments (Sondi and Salopek-Sondi 2004; Oberdorster *et al.* 2006; Lyon *et al.* 2006; table 1.2).

Table 1.2. Examples for nanoparticle (eco-) toxicity and other effects.

Toxicity study	*Nanotype*	*Reference*
In vitro cytotoxicity of oxide nanoparticles	SiO_2, Fe_2O_3, TiO_2, ZnO, $Ca_3(PO_4)_2$, CeO_2, ZrO_2	Brunner *et al.* 2006
Tissue sites of uptake of ^{14}C-labelled C60	C60	Bullard-Dillard *et al.* 1996
Cytotoxicity of quantum dots	Quantum dots	Shiohara *et al.* 2004, Hardman 2006
Transport of surface-modified nanoparticles through cell monolayers	Amino-CLIO	Koch *et al.* 2005
Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles	SiO_2	Chen and von Mikecz 2005
Manufactured nanomaterials (Fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass	C60	Oberdorster 2004
Daphnia magna mortality when exposed to titanium dioxide and fullerene (C60) nanoparticles	C60, TiO_2	Lovern and Klaper 2006
Phytotoxicity of alumina nanoparticles	Alumina	Yang and Watts 2005
Silver nanoparticles as antimicrobial agent	Silver	Sondi and Salopek-Sondi 2004
Antibacterial activity of fullerene water suspensions	C60	Lyon *et al.* 2006

Even in cases where nanoparticles do not show any acute toxicity, questions of long-term effects, bioaccumulation and the impact on food webs remain unanswered. Engineered nanoparticles may also affect the toxicity of other substances, since natural nanomaterials are known to act as nanovectors for contaminants (Mccarthy and Zachara 1989; Kersting *et al.* 1999; Lyven *et al.* 2003; Lamelas and Slaveykova 2007). For example, a study on carp showed enhanced cadmium bioaccumulation in the presence of TiO_2 nanoparticles (Zhang *et al.* 2007).

Therefore, it is crucial that we begin to understand the behaviour of engineered nanoparticles in food materials, consumer products and environmental matrices, as well as their toxicity to humans and the environment. To accomplish this, access to robust analytical methodologies is essential for detecting and characterizing engineered nanoparticles in a range of matrix types.

The primary aims of this study were to therefore (a) develop and evaluate analytical methods for quantifying, visualising engineered nanoparticles in a range of environmental matrices; (b) to apply the developed techniques to begin to understand the fate of engineered nanoparticles in activated sludge treatment systems; and (c) based on the knowledge gained, to provide recommendations on how the environmental risks of engineered nanoparticles could be better assessed in the future. These aims were achieved using the following specific objectives:

1. Review available analytical methods potentially suitable for the detection of ENPs in complex matrices
2. Test the most promising and novel analytical methods as identified in 1. for the detection and characterisation of metal-based ENPs in aquatic systems
3. Develop novel analytical methods for metal-based ENP detection and characterisation in aquatic systems and validate the applicability of developed approaches for experimental fate studies
4. To use the developed methods to explore the fate of selected ENPs in the aquatic environment

5. Using data from 4. alongside information on usage and usage patterns of the study ENPs, quantify the likely magnitude of environmental exposure and explore the subsequent risks to aquatic communities
6. Based on the knowledge developed in 1. – 4., provide recommendations on how environmental fate and effect studies could be better designed in order to ensure more accurately characterise the risks related to ENPs

In this thesis solely metal and metal oxide ENPs are studied as they are currently used in a range of products (paints, sunscreens, cosmetics etc.) and their use pattern is expected to give rise to environmental exposure (Hansen *et al.* 2007b). Also, metal and metal oxide ENPs are commercially available in a range of sizes.
Additionally, the analysis in complex matrices is likely to be less challenging than analysis of carbon-based ENPs and knowledge gained could assist in developing methods for carbon-based ENPs in the future.

The aims and objectives described above have been addressed in 5 stand-alone papers, which constitute the main part of this thesis:

Chapter 2 is a critical review of existing analytical methods to detect and characterise ENPs in food and the environment. Selected methods are illustrated using images generated during experiments performed during this PhD programme. The most promising methods for engineered nanoparticles characterisation in the aquatic environment were identified and the novel wet scanning electron microscopy (WetSEM^{TM}) technology was selected for further assessment (chapter 3). Further, the need to develop new analytical methods was identified and addressed in chapter 4.

In *chapter 3* the suitability of sub-micrometer microscopic techniques for the visualisation and characterisation of metal-based ENPs in aquatic systems is discussed. Conventional scanning electron microscopy (SEM) and the novel approach of WetSEM^{TM}, enabling particle imaging under fully liquid conditions, are compared.

In *chapter 4* the development of hydrodynamic chromatography inductively coupled plasma mass spectrometry (HDC-ICP-MS) is described for the simultaneous size characterisation and elemental analysis of metal-based ENPs in aquatic systems. Gold nanoparticles were assessed as size calibration standards and relative retention time markers. The method was validated for a range of different inorganic nanoparticles in water and sewage sludge.

In *chapter 5* this novel HDC-ICP-MS method is applied in a laboratory-based study to assess the fate of nanosilver as a model metal-based nanoparticle in activated sewage sludge processes. Results were confirmed by transmission electron microscopy. The obtained data was used alongside simple exposure models to predict the potential nanosilver concentration resulting from water treatment works in surface waters and therefore to support environmental risk assessment of ENPs.

The experience and knowledge gained from the analytical development work and fate investigations is used in *chapter 6* to discuss how environmental fate and effects studies could be better designed in the future in order to more accurately assess the risks of ENPs to the environment.

Chapter 7 provides overall conclusions and recommendations for future research.

Chapter 2

Review: Detection and characterisation of engineered nanoparticles in food and the environment

Introduction

This chapter provides an overview of the different analytical techniques available for the detection as well as physical and chemical characterisation of engineered nanoparticles in product formulations, environmental matrices and food materials. As limited work has been done to date on the detection of engineered nanoparticles in the environment and food, this review draws heavily on studies reporting detection and characterisation of nanoparticles in raw products and natural nanoparticles in environmental systems where much more information is available (*e.g.* Walther 2003; Lead and Wilkinson 2006; Wigginton *et al.* 2007a). Possible future directions of ENP analysis and characterisation in biological, environmental or food samples are identified and areas of further research are recommended.

Nanoparticle properties & their analysis

The potential toxicity and behaviour of nanoparticles will be affected by a wide range of factors including particle number and mass concentration, surface area, charge, chemistry and reactivity, size and size distribution, state of aggregation, elemental composition, as well as structure and shape (Borm and Muller-Schulte 2006; Chau *et al.* 2007; table 2.1). Therefore, when analysing nanoparticles in different matrices, it is not only the composition and concentration that will need to

be determined but also the physical and chemical properties of the engineered nanoparticles within the sample and the chemical characteristics of any capping/functional layer on the particle surface.

The analytical techniques should be sensitive enough to measure low concentrations, as small particles normally represent only a small part of the total mass. The techniques should also minimise sample disturbance to ensure that laboratory analyses reflect the unperturbed environmental state (Chen and Buffle 1996; Gimbert *et al.* 2007). A range of analytical techniques is available for providing information on concentration and properties, including microscopy approaches, chromatography, centrifugation and filtration, spectroscopic and related techniques (table 2.2). In the following parts of this chapter, a selection of these methods will be discussed that are potentially suitable for nanoparticle characterisation and literature examples will be used to demonstrate the application of different techniques to complex media.

Overview of analytical methods applicable to nanoparticle analysis

A wide range of methods is available for the detection and characterisation of nanoparticles; a choice of different approaches are described below and a summary of the information generated by different techniques and their application to complex media is given in table 2.2 and table 2.3, respectively.

Table 2.1. Nanoparticle properties and their importance for measurement.

Property	*Importance of measurement*
Aggregation state	Nanoparticles that have a tendency to aggregate and are bigger than 100 nm in their aggregated state are not classed as nanoparticles
Elemental composition	Different particle composition leads to different behaviour/impact, *e.g.* Cd vs Fe
Mass concentration	Normally increased contaminant concentration leads to increase in toxicity/impact, this is not always applicable for nanoparticles
Particle number concentration	Nanoparticles have low mass concentrations, but show high percentage of total particle numbers
Shape	Different particle shapes (*e.g.* spherical, tubular) can posses different affinities or accessibilities *e.g.* transport through membranes into cells, different antibacterial behaviour
Size & size distribution	Nanoparticles are defined and classed by their size and size is one of the primary properties describing transport behaviour
Solubility	Soluble nanoparticles; once dissolved cannot be classed as nanoparticles (*e.g.* ZnO vs Zn^{2+})
Speciation	Different species can have different behaviour, toxicity, impact (*e.g.* C60 vs C70, ENP complexes with natural organic matter or oxidation state)
Structure	The structure can have an influence on stability or behaviour (*e.g.* rutile or anatase as possible crystal structures of TiO_2)
Surface area (& porosity)	Increase in surface area increases reactivity and sorption behaviour
Surface charge	Surface charge has an influence on particle stability especially in dispersions
Surface chemistry	Coatings can consist of different chemical compositions and influence particle behaviour or toxicity (*e.g.* Quantum dots with CdSe core and ZnS shell)

Table 2.2. Nanoparticle properties and examples of analytical methods potentially suitable for their measurement (definitions of acronyms are given in the text, list of acronyms and table 2.3).

Nanoparticle properties	*Microscopy and related techniques*	*Chromatography and related techniques*	*Centrifugation and filtration techniques*	*Spectroscopic and related techniques*	*Other techniques*
Aggregation	*e.g.* STEM, TEM, SEM, AFM. STM		*e.g.* ANUC	*e.g.* XRD, SANS	*e.g.* Zeta potential
Chemical composition	AEM, CFM			*e.g.* NMR, XPS, Auger, AES, AAS, MS, XRD, EBSD	
Mass concentration	AEM, CFM	√		√	*e.g.* Gravimetry, thermal analysis
Particle number concentration					*e.g.* Particle counter, CPC
Shape	*e.g.* STEM, TEM, SEM, AFM. STM	*e.g.* FlFFF-SLS, SedFFF-DLS	*e.g.* UC		
Size	*e.g.* STEM, TEM, SEM, AFM, STM	√			*e.g.* DMA
Size distribution	*e.g.* STEM, TEM, SEM, AFM, STM	*e.g.* FFF, HDC, SEC	*e.g.* CFF, UC, CFUF	*e.g.* SPMS, SAXS	*e.g.* UCPC, SMPS
Dissolution			Dialysis, CFUF		Voltammetry, diffusive gradients in thin films
Speciation		*e.g.* SEC-ICP-MS		*e.g.* XAFS, XRD	*e.g.* Titration
Structure	*e.g.* STEM, TEM, SEM, AFM, STM			*e.g.* XRD, SANS	
Surface area (& porosity)					*e.g.* BET
Surface charge		*e.g.* CE			*e.g.* Zeta potential
Surface chemistry	AEM, CFM			*e.g.* XPS, Auger, SERS	

Table 2.3. Overview of discussed analytical methods suitable for nanoparticle characterisation in alphabetical order with literature examples for their application in complex media.

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
3D fluorescence excitation-emission matrix	EEM	ppb		Complex data interpretation	Probing chemical structure / functional groups		Fluorescent characteristics of colloidal organic matter filtrates	Liu *et al.* 2007
Aerosol time of flight mass spectrometry	ATOFMS	3 nm - µm particle size	Analysis of individual particles Real time measurement	Not fully quantitative	Sizing Chemical composition		Single particle analysis Aerosols	Angelino *et al.* 2001 Prather *et al.* 1994 Suess and Prather 1999
Analytical electron microscopy (EDX&EELS)	AEM	~ 0.5 nm	*e.g.* EELS also applicable for light elements (< Zn)	*e.g.* EDX only applicable for heavier elements	Chemical composition (Semi-) quantitative analysis	TEM SEM STEM	Combination of electron microscopy with AEM techniques like EELS and EDS	Mavrocordatos and Perret 1998 Leppard *et al.* 2004 Luther 2004 Gilbert *et al.* 2004

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Atomic force microscopy	AFM	~ 0.1 nm	Dry, moist or liquid samples, ambient environment 3D surface profiles, sub nanometre topography resolution	Overestimations of lateral dimensions, artefacts due to movement of particles(smearing) and particles adhering to the tip	Sizing Electrical and mechanical properties Visualization		Force measurement between sample and tip CFM = chemical force microscopy, Quantum electronic mapping: STM=scanning tunnelling microscopy	Lead *et al.* 2005 Friedbacher *et al.* 1995 Maurice 1996 Bickmore *et al.* 1999 Balnois *et al.* 1999 Balnois and Wilkinson 2002 Yang *et al.* 2007 Wigginton *et al.* 2007a
Auger electron spectroscopy	AES	~ 1 – 2 nm			Surface composition Surface topography Oxidation state	SEM	Extremely surface sensitive technique	Powell and Seah 1980 Liu 2005
Brunauer Emmett Teller	BET	Thousands of m^2/g			Total surface area Porosity			Brunauer *et al.* 1938 Nurmi *et al.* 2005

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Capillary electrophoresis	CE		Sensitive, fast, & separation by charge	Mobile phase interactions, complex data interpretation, need of standard material	Electrophoretic mobility Sizing Separation of ionic species by charge and frictional forces	UV/Vis Fluo MS		Schmitt-Kopplin and Junkers 2003 Chan *et al.* 2007 Lin *et al.* 2007
Centrifugation		For a given density and spherical particles: what is the size ranges for a certain number of g	Low surface effects	Aggregation can be induced by differential settling velocity (heavier, larger particles bump into slower settling velocities)	Settling rates, buoyant mass, for known density: equivalent spherical volume, size separation		*e.g.* differential centrifugation	Lead *et al.* 1999 Novak *et al.* 2001 Bootz *et al.* 2004 Lyon *et al.* 2006
Condensation particle counter	CPC				Number concentration	DMA		Luther 2004 Flagan and Ginley 2006
Cross flow ultrafiltration	CFUF	1 nm – 1 μm	Higher speed, higher volume, less concentration polarisation and clogging than piston filtration or stirred cells	Potential alterations, due to increased particle concentrations, turbulent flows, extensive surface exposure Not well defined size fractionation	Separation based on size & surface charge			Guo *et al.* 2000 Doucet *et al.* 2004 Doucet *et al.* 2005b Liu and Lead 2006 Sung *et al.* 2007

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Cryo transmission electron microscopy	Cryo-TEM		Imaging of liquid & biological specimen	Sample alteration	Sizing Visualization	EDS	Special sample holder needed	Guo *et al.* 2000 Tang *et al.* 2004
Differential mobility analyser	DMA	3 nm - µm particles	In combination with a wide range of techniques	For water necessary to form an aerosol that is dried in which can cause sample changes	Sizing	ES CPC ICP-OES ICP-MS ATOF-MS	Also as tandem differential mobility analyser (TDMA)	McMurry *et al.* 1996 Weber *et al.* 1996 Cass *et al.* 2000 Seol *et al.* 2001 Okada *et al.* 2002 Luther 2004 Flagan and Ginley 2006 Naono *et al.* 2006

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Dynamic light scattering (photon correlation spectroscopy or quasi elastic light scattering)	DLS (PCS, QELS)	3 nm - µm particles	*In situ* measurement Rapid and simple analysis, useful to follow aggregation processes	Difficult to interpret results based on intensity weighted sizes. Aggregates dust particles can ruin the measurements on nanoparticles Multiple scattering and particle interactions in high concentrations, limited capability on polydisperse samples.	Intensity weighted diffusion coefficient can be calculated to a z-average hydrodynamic diameter or distribution			Huve *et al.* 1994 Bootz *et al.* 2005 Lecoanet *et al.* 2004 Lecoanet and Wiesner 2004 Brant *et al.* 2005a Phenrat *et al.* 2007 Viguie *et al.* 2007
Electrophoretic mobility	EM	>3nm	Minimum perturbing, rapid and simple measurement	Interpretation of the zeta potential in relation to surface potential	Net Zeta potential (potential at a slipping plane in the electric double layer of the particle)	DLS	Dependence of electrolyte solution	Ryan *et al.* 2000 Lecoanet *et al.* 2004 Brant *et al.* 2005b Chen and Elimelech 2007 Reiber *et al.* 2007

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Electro-zone sensing					Sizing Number concentration Surface charge			Ito *et al.* 2003
Environmental scanning electron microscope	ESEM	30-50 nm	No sample preparation No charging effects Variable temperature & pressure Imaging of hydrated samples	Loss in resolution Contrasting Atmospheric pressure & imaging under fully wet conditions not possible	Sizing Chemical composition Visualization	EDS	Semi-*in situ* measurements	Bogner *et al.* 2005 Redwood *et al.* 2005 Doucet *et al.* 2005a De Momi and Lead 2006

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Field flow fractionation	FFF	Flow FFF 1 nm – 1 µm Sed FFF: 50nm-1µm	Size range, mild fractionation, direct relation between retention time and size, versatility in carrier composition	Optimization of carrier composition demands experience, membrane interactions, dilution, concentration gradients	Size distributions (Flow FFF: diffusion coefficient and hydrodynamic diameter, Sed FFF: buoyant mass and equivalent spherical diameter) Size separation	On-line: UV/Vis DRI MALLS ICP-MS FLD LIBS Off-line: TEM-EDS AFM		Baalousha *et al.* 2006a Baalousha *et al.* 2006b Baalousha and Lead 2007 Beckett and Hart 1993 Gimbert *et al.* 2006 Gimbert *et al.* 2005 Gimbert *et al.* 2007 von der Kammer *et al.* 2005 Lyven *et al.* 2003 Rameshwar *et al.* 2006 Siepmann *et al.* 2004 Siripinyanond *et al.* 2005 Stolpe *et al.* 2005 Lyven *et al.* 1997 Siripinyanond *et al.* 2002

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Filtration			Fast Low cost	Clogging	Size separation			Kang and Shah 1997 Lau *et al.* 2004 Marani *et al.* 2004 Hett 2004
Fluorescence correlation spectroscopy (Confocal microscopy)	FCS	~ 200 nm	Dilute samples in small volumes No multiple scattering	Only fluorescent samples	Diffusion coefficient, hydrodynamic diameter, Concentration	Fluorescence labelling		Kuyper *et al.* 2006b Kuyper *et al.* 2006a Pinheiro *et al.* 2007 Lead *et al.* 2000b
High performance liquid chromatography	HPLC			Mobile phase interactions Size separation range limited by column	Sizing Separation Purification Quantification	UV/Vis ICP-MS Voltammetry Amperometry		Scrivens *et al.* 1994a Sivamohan *et al.* 1999 Song *et al.* 2004 Song *et al.* 2003 Giusti *et al.* 2005
Hydrodynamic chromatography	HDC	5 – 1200 nm		Mobile phase interactions	Sizing Size separation	UV/Vis ICP-MS		Blom *et al.* 2003 Williams *et al.* 2002 Yegin and Lamprecht 2006
Laser induced break down diffraction	LIBD		No sample preparation Solid phase elemental analysis	Destructive Interferences with ambient environment	Chemical composition	OES		Heuser and Walker 2004

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Membrane filtration		Mainly 0.2 & 0.4 μm filtration steps	High speed, high volume fractionation	Broad pore size distribution. Filtration artefacts	Size separation			Akthakul *et al.* 2005 Howell *et al.* 2006
Mössbauer spectroscopy	Mössbauer				Oxidation state Phase identification Magnetic properties		Bulk	Burleson *et al.* 2004
Near-field scanning optical microscopy	NSOM	~ 30 nm	Optical imaging	Spatial resolution	Sizing Chemical bonding Visualization		Thin samples ~ 200 nm	Maynard 2000
Nuclear magnetic resonance spectroscopy and Pulsed field gradient NMR	NMR		Suitable for colloidal matter in liquid or solid state	Lack of available standards	PFG-NMR: diffusion coefficient hydrodynamic diameter, Structure of coating & particles Chemical composition			Valentini *et al.* 2004 Luther 2004 Carter *et al.* 2005
Raman spectroscopy	Raman		Compatible with aqueous suspensions & wet nanoparticle samples	Parameter effects	Oxidation state Structure Sizing		Vibrational spectroscopy Bulk	Li Bassi *et al.* 2005

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Scanning electron microscopy	SEM	1 nm – 1 µm	High resolution	High vacuum Sample preparation Contrasting Charging effects	Sizing	Auger EDS		Paunov *et al.* 2007
Scanning mobility particle sizer	SMPS				Size distribution Sizing Number concentration			Hasegawa *et al.* 2004 Luther 2004 Lenggoro *et al.* 2007
Scanning transmission electron microscopy	STEM	< 0.1 nm	Analysis of low concentrations (ppm)		Sizing Shape Structure Visualization	XRD HAADF CEND ADF TAD AEM CBED		Utsunomiya and Ewing 2003 Liu 2005 Bogner *et al.* 2005
Scanning transmission X-ray microscopy	STXM	30 nm	No sample preparation, liquid conditions		Sizing Shape Visualization			Leppard *et al.* 2004 Nurmi *et al.* 2005 Thieme *et al.* 2007
Secondary ion mass spectrometry	SIMS		Atomic composition of layers from 1 – 3 nm	Sample preparation Offline technique Destructive	Chemical composition Surface properties			Kim *et al.* 1999 Borm *et al.* 2006

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Single particle mass spectrometer	SPMS				Sizing Chemical composition			Janzen *et al.* 2002 Cai *et al.* 2002 Lee *et al.* 2005
Size exclusion chromatography	SEC		Good separation efficiency, simple	Unwanted solvent & column interactions Limited size separation range	Separation Sizing	DRI FL PDA UV/Vis ICP-MS		Bolea *et al.* 2006 Huve *et al.* 1994 Krueger *et al.* 2005 Novak *et al.* 2001 Zhou *et al.* 2000 Zhao *et al.* 2001 Wilcoxon and Provencio 2005 Helfrich *et al.* 2006 Wang *et al.* 2006
Small angle neutron scattering	SANS		Analysis in liquids		Charge density Structure in dependence of pH, ionic strength, solute concentration			Diallo *et al.* 2005

Table 2.3. ***Continued.***.

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
Static light scattering	SLS				Molecular weight Root mean square radius of gyration	SEC FFF DLS		Baalousha *et al.* 2005a Baalousha *et al.* 2005b
Thermo-gravimetric analysis	TGA				Oxidation state		Bulk analysis	Pang *et al.* 1993
Time-of-flight mass spectrometry	TOF-MS	ppb-ppt			Mass/charge ratio Chemical composition	Other TOF-MS variations: LAI MALDI NAMS	Aerosols Macromolecules like polymers	Lou *et al.* 2000 Reents *et al.* 1995 Wang and Johnston 2006 Lou *et al.* 2000
Transmission electron microscopy	TEM	> 0.1 nm	High resolution	Sample preparation High vacuum Contrasting	Sizing Shape Visualization Structure	EELS EDS		Mavrocordatos and Perret 1998 Wilkinson *et al.* 1999 Mavrocordatos *et al.* 2004
Ultracentrifugation (analytical/ preparative)		Size range: 100 Da to 10GDa (molar mass from calibrations)	Acceleration: up to 1,000,000 G (9,800km/s^2)	Differential settling rates can induce aggregation	Sedimentation velocity Sedimentation equilibrium Shape and molar mass Size distribution			Bootz *et al.* 2004

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
UV/Vis spectroscopy	UV/Vis		*In situ*	Insensitive	Quantitative Concentration, some structure or size information can be derived			Pesika *et al.* 2003
Wet scanning electron microscopy	WetSEM	Low contrast samples: ~ 100 nm High contrast samples: ~ 10 nm	Imaging under fully wet conditions	Loss in resolution Sensitive membrane	Sizing Shape Visualization	EDS	Wet imaging	Timp *et al.* 2007
Wet scanning transmission electron microscopy	WetSTEM		Imaging in liquids		Sizing Shape Visualization		Transmission observations in ESEM	Bogner *et al.* 2005
X-ray absorption spectroscopy	XAS	ppm			Oxidation state Chemical composition Structure		Includes EXAFS and XANES Bulk	Venkateswarlu *et al.* 2005 Arcon *et al.* 2005
X-ray diffraction	XRD	1 – 3 wt%			Structure Sizing		Especially for crystalline nanoparticles Bulk	Zhang *et al.* 2003 Guzman *et al.* 2006a

Table 2.3. ***Continued.***

Method	*Acronym*	*Spatial resolution or LOD*	*Advantages*	*Disadvantages*	*Information*	*Possible combination*	*Comments*	*Examples of application/References*
X-ray fluorescence spectroscopy	XRF				Solid state speciation Quantitative bulk analysis Isotope ratios Morphology		Aerosols	Ortner *et al.* 1998
X-ray microscopy	XRM	~ 30 nm		Radiation damage	Sizing Shape Visualization			Jearanaikoon and Braham-Peskir 2005
X-ray photoelectron spectroscopy	XPS	~ 1 μm	Atomic composition of layers from 1 – 10 nm		Shape Sizing Chemical composition Oxidation state		Extremely surface sensitive technique	Schrick *et al.* 2004 Nurmi *et al.* 2005

Microscopy and microscopy related techniques

Microscopy-based methods include optical approaches, *i.e.* confocal microscopy, as well as electron and scanning probe microscopy.

The typical dimensions of nanoparticles are below the diffraction limit of visible light, so that they are outside of the range for optical microscopy. However, near-field scanning optical microscopy (NSOM) – a scanning probe microscopy (SPM) technique – can obtain a spatial resolution of ~ 50-100 nm, much better than conventional optical microscopes. This is achieved through the use of a sub-wavelength diameter aperture. NSOM may, therefore, be suitable for optical imaging of nanoparticle aggregates (Maynard 2000).

The diffraction of light is also the limiting factor for confocal microscopy. However, using confocal laser scanning microscopy (CLSM), resolutions of up to 200 nm can be achieved and tiny fluorescent objects can often be located more precisely than the resolution limit. Another feature of a CLSM is the high-resolution optical imaging of thick specimens (optical sectioning). Naturally fluorescent samples or samples treated with fluorescent dyes are detectable. Confocal microscopy has only recently been applied in colloid characterisation and has been combined with fluorescence correlation spectroscopy (FCS) to characterize fluorescent species in complex systems (Lead *et al.* 2000b; Prasad *et al.* 2007).

The most popular tools for the visualization of engineered nanoparticles are electron and scanning probe microscopes. Depending on the technique, resolutions down to the sub-nanometre range can be achieved. Using atomic force microscopy (AFM), scanning electron (SEM) and transmission electron microscopy (TEM), nanoparticles can not only be visualized, but also properties such as the state of aggregation, dispersion, sorption, size, structure and shape can be observed (Mavrocordatos *et al.* 2004). For comparison, figure 2.1 shows TiO_2 and ZnO nanoparticles imaged by SEM, TEM and AFM (all images shown were obtained by the candidate; AFM images obtained in tapping mode).

In TEM, electrons are transmitted through a specimen (therefore, the specimen has to be very thin) to obtain an image; in SEM scattered electrons are detected which originate from the surface of the sample thus imaging the sample's surface topography. In general, imaging samples comprising lighter atoms, compared to

higher mass atoms, in an electron microscope is more demanding as less contrast is produced in the image due to reduced electron scattering.

Analytical (mostly spectroscopic) tools can be coupled to electron microscopes for additional elemental composition analysis, generally known as analytical electron microscopy (AEM). For example, energy dispersive X-ray spectroscopy (EDS) can be combined with SEM and TEM permitting a clear determination of the composition of elements heavier than oxygen, quantitative analysis, however, leads to a ~ 20 % uncertainty (Mavrocordatos *et al*. 2004).

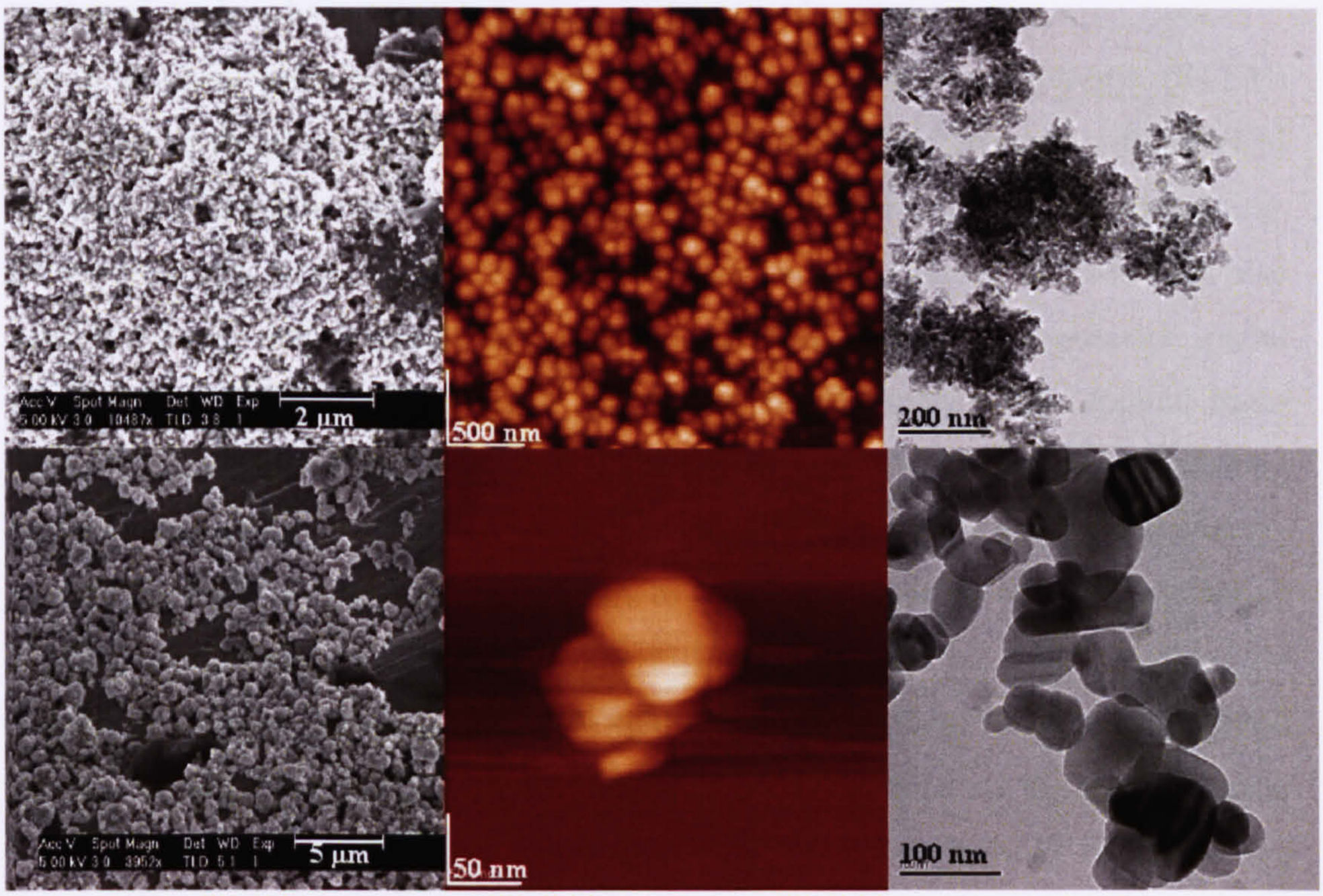


Figure 2.1. ZnO (1st row) and TiO_2 (2nd row) nanoparticles suspended in distilled water, allowed to dry and imaged in order from left to right by SEM, AFM and TEM. Initial sizes as stated by the manufacturer (Sigma Aldrich, UK): 50 – 70 nm for ZnO particles and 5 – 10 nm for TiO_2 particles (own work).

Electron energy loss spectroscopy (EELS) is based on characteristic energy losses in the incident electron as it passes through the specimen due to interactions with individual atoms within the sample. The specific energy losses observed enable identification of the elements within the specimen. This technique can only be used with TEM and quantitative analysis has uncertainties as low as 10 % (Mavrocordatos

et al. 2004). Selected area electron diffraction (SAED) can also be combined with TEM and provides information on crystalline properties of particles (Mavrocordatos *et al.* 2004).

Electron microscopy is usually a destructive method, meaning that the same sample cannot be analysed twice or by another method for validation. Other disadvantages of electron microscopes are charging effects caused by accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. This can normally be overcome by using a sample coating made of a conducting material, but this can result in a loss of information. Also, biological samples often need treatment, such as heavy metal staining, for improved contrast.

For biological samples, a scanning transmission electron microscope (STEM) belonging to the group of TEMs can be of use. Dark-field microscopy with a STEM allows high contrasts and, therefore, imaging of biological samples without staining. In combination with diffraction and spectroscopic techniques, STEMs can also provide images and chemical data for nanomaterials with a sub-nanometre spatial resolution (Liu 2005). Utsunomiya and Ewing (2003) successfully applied high-angle annular dark-field scanning transmission electron microscopy, scanning transmission electron microscopy-energy dispersive X-ray spectrometry and energy-filtered transmission electron microscopy to the characterisation of heavy metals on airborne particulates.

X-ray microscopy (XRM) can provide spatial resolution (down to ~ 30 nm, limited by the X-ray beam focusing optics) imaging of a specimen in the aqueous state without the need for sample preparation, *e.g.* fixation, staining or sectioning (Jearanaikoon and Braham-Peskir 2005; Thieme *et al.* 2007). X-ray microscopy can also be combined with computer tomography to enable 3D imaging (Thieme *et al.* 2003). A variation of the XRM is the scanning transmission X-ray microscopy (STXM), which has been used, for example, to characterize metallic Fe particles for remediation purposes (Nurmi *et al.* 2005).

The major limitation of conventional electron microscopes, such as transmission electron and scanning electron microscopes, is that they have to be operated under vacuum conditions. This means no liquid samples can be introduced to the sample chamber and sample preparation (dehydration, cryofixation or embedding) is

necessary, which usually leads to sample alteration and dehydration artefacts (Mavrocordatos *et al.* 2007).

To limit artefacts, efforts have been made to improve sample preparation techniques for electron microscope imaging. For example, Lonsdale *et al.* (1999) applied high pressure freezing and freeze substitution to image barley aleurone protoplasts by transmission electron microscopy (TEM). This method preserves the cellular fine structure and antigenicity of proteins better than conventional chemical fixation and dehydration techniques. Another possibility is the use of a cryo-TEM, which enables imaging of frozen samples on a cold specimen-stage and microscope. This has the advantage of preserving and visualizing structures that would be lost or altered by other sample preparation methods. Wang *et al.* (2004) employed this method to image Fe(III)-doped TiO_2 nanoparticles (2-4 nm) in an aqueous environment with a special sample holder. Mavrocordatos and Perret (1998) embedded iron-rich particles (30-200 nm) in resin and then sectioned these samples for visualization by TEM and EELS.

However, none of these preparative techniques can fully avoid artefacts caused by sample drying or preparation. As imaging of nanoparticles in their original state is crucial for nanoparticle research, other methods are required. One possibility to image nanoparticles under more natural conditions is to use an environmental scanning electron microscope (ESEM). In an ESEM, the gun and lenses of the microscope are under vacuum conditions as in a conventional SEM, but, due to a detector that is able to operate under higher pressure and multiple pressure limiting apertures to separate the sample chamber from the column, the sample chamber itself can be operated at around 10-50 Torr. Therefore, samples can theoretically be imaged in their natural state without modification or preparation under variable pressure and humidity, theoretically up to 100 %. Additionally, the gas ionization in the ESEM sample chamber eliminates the charging artefacts and, therefore, materials no longer have to be coated with a conducting material. Other advantages of an ESEM are that the detector is insensitive to light, and fluorescence or cathodoluminescence does not disturb imaging. ESEM still allows X-ray data, *e.g.* from EDS, to be obtained. However, an ESEM cannot achieve real atmospheric pressure and only the top surface of a specimen can be imaged, which, in the case of

a liquid sample, is the water surface. The contrast is increasingly poor with increasing humidity and there is the possibility of specimen drifting. Also, a loss in resolution from ~ 10 nm up to ~ 100 nm is unavoidable.

Doucet *et al.* (2005a) compared the performance of an environmental and a conventional scanning electron microscope (ESEM and SEM, respectively) for the imaging of natural aquatic particles and colloids. Analysing river estuary samples they found that the conventional SEM provides sharper images and lower resolution limits, but produces more imaging artefacts due to drying of the sample. To some extent, ESEM samples retain their morphological structures without the need of sample preparation, but image interpretation and imaging itself is more complex. Also, it has been stated that the maximum relative humidity at which imaging could be performed was 75 %, as, at 100 %, layers of free water over the sample made colloid visualization impossible. Sizing of colloids revealed technique-dependent differences; hence, Doucet *et al.* (2005a) suggest that ESEM and SEM should be used as complementary techniques, but are in favour of the ESEM for imaging colloids and colloid aggregation. Redwood *et al.* (2005) applied an ESEM to analyse and quantify humic substances (Suwannee river humic acid, 100 mg/L) as a function of humidity and pH (3.3-9.8). They concluded that ESEM is an important complementary technique to other analytical methods for probing changes in colloid structure as a function of hydration state; however, they also concluded that at present non-perturbed samples cannot be imaged (Redwood *et al.* 2005).

The technique of WetSTEM allows transmission observations of wet samples in an ESEM under annular dark-field imaging conditions down to a few tens of nm. Combining elements of TEM and ESEM, samples that are fully submerged can be imaged. The imaging is achieved by placing a TEM grid with the sample on a TEM sample holder. This holder is placed in the ESEM chamber allowing transmission imaging under non-vacuum conditions (Bogner *et al.* 2005).

An alternative to the ESEM methods described above is the use of a WetSEM^{TM} capsule as a specimen holder, in which the sample is added and the holder is then sealed. These capsules have been developed by QuantomiX (Rohovot, Israel) for imaging of samples in a conventional SEM under hydrated conditions. There are two different types of WetSEM^{TM} capsules on the market suitable for conventional SEM

with a back-scattered electron detector: one for imaging in liquids and another for imaging of solid but wet materials (*e.g.* biological samples, food or soil). With this technique, *in situ* imaging of nanoparticles in natural media is possible. The capsule separates the sample from the vacuum chamber of the microscope and a membrane in the capsule allows electrons to pass into the sample; thus, enabling imaging under atmospheric pressure. It is possible to conduct semi-quantitative and qualitative elemental analysis with these capsules provided the microscope is equipped with an energy dispersive X-ray spectrometer (Thiberge *et al.* 2004b; Thiberge *et al.* 2004a; Joy and Joy 2006; Timp *et al.* 2007). Limitations are a loss of resolution and the sensitivity of the membrane to radiation damage. Also, objects have to be close to the membrane to be visible. Thiberge *et al.* (2004a & b) describe in detail the theory, characteristics, limitations and possible applications of WetSEM^{TM} capsules using a conventional SEM and an ESEM. There are no studies present in the literature on the use of WetSEM for studying nanoparticles in environmental conditions.

Imaging under fully liquid conditions is also possible using atomic force microscopy (AFM). The AFM belongs to the family of scanning probe microscopes (SPMs) (Balnois *et al.* 2007). An oscillating cantilever is scanning over the specimen surface and electrostatic forces (down to 10^{-12} N) are measured between the tip and the surface. An AFM can achieve 3D surface profiles from these force measurements with height resolutions of ~ 0.5 nm. The main advantage of an AFM is that it images sub-nanometre structures under wet or moist conditions. Although under liquid conditions particles not fixed to a substrate will float around and eventually stick to the cantilever, which leads to imaging artefacts, both as smearing effects and changes in the cantilever oscillation properties, as the tip gains weight. This smearing effect could be minimized by using a non-contact scanning mode where the tip is not touching the particles but only feel its forces (Balnois *et al.* 2007). The main limitation of AFM for nanoparticle visualization is that the geometry of the tip is often larger than the particles being probed and this leads to errors in the onset and offset of particle topography on a scan, resulting in severe overestimations of the lateral dimensions of the nanoparticles. Therefore, accurate size measurements should only be taken on the height (z-axis) of the particles and the lateral dimensions only used with great caution. Furthermore, AFM for environmental or food related

samples is limited in the ability to obtain qualitative or quantitative information of the sample composition. Nevertheless, the force patterns that emerge can also help in identifying the nature of individual atoms via a technique called chemical force microscopy (CFM) (Sugimoto *et al.* 2007; Shluger and Trevethan 2007). This recent development could lead to progress in AFM application to more complex samples. Scanning tunnelling microscopy (STM) is another type of scanning probe microscopy and is based on the quantum mechanical nature of electrons on the sub-nanometre scale. A conducting tip is brought into proximity of a metallic or semi conducting surface such that when the gap between the surface and the tip is ~ < 1 nm, and a small voltage applied to the tip (or surface), electrons can 'tunnel' through this gap creating a very small current, the magnitude of which is very sensitive to the tip-surface separation. By scanning the tip across the surface and adjusting the height of the tip to maintain a constant tunnelling current, the surface can be imaged with a resolution of ~ 1 nm or better. STM has been applied to environmental samples to image redox properties of microbial enzymes (Wigginton *et al.* 2007b).

AFM has been used to characterise natural colloidal matter. For example, Lead *et al.* (2005) analysed natural aquatic colloids by AFM and their structure was found to vary as a function of pH. Mica slides were dipped for 30 min into filtered samples rinsed with distilled water and allowed to dry prior to imaging in tapping mode. It has been stated that it is not known whether imaging under ambient humidity or liquid water produces better results. *A priori*, imaging under liquid water appears to provide ideal experimental conditions. However, atmospheric humidity retains colloid-bound water, helping to maintain structure, and AFM tips exposed to organic matter in solution soon become coated in the organic matter, potentially affecting the veracity of the images. This is also a possibility in imaging after air-drying. Comparing TEM and AFM using different sample preparation methods indicated similar morphologies (Lead *et al.* 2005). Balnois *et al.* (1999) employed tapping mode AFM for the analysis of humic acid on mica. They found that aggregation might be related to the hydrophobicity of the sample. No aggregates were observed for relatively hydrophilic humic acids (Suwannee river) at pH 3-10, but aggregates were seen for peat humic acid at low pH and high ionic strength. A comparison

between AFM, Fluorescence Correlation Spectroscopy, Field-Flow Fractionation and Pulsed Field Gradient-NMR on a reference fulvic acid sample (Lead *et al.* 2000a) consistently showed that AFM resulted in smaller particle sizes measurements than the other techniques, even though AFM is a number-average method whereas the others are mass-average methods. This underestimation of the size of the fulvic acid was thought to be due to drying or other substrate effects during the AFM procedure. Although an AFM is operated under ambient conditions, samples still have to be applied to a specimen holder, which can cause alterations; thus, sample application has to be done carefully. A range of sample preparation techniques have been reported by Balnois and Wilkinson (2002), including drop deposition, adsorption, ultracentrifugation, which have successfully been applied in the characterisation of environmental biopolymers (*e.g.* humic substances, polysaccharides) by AFM (Balnois and Wilkinson 2002). Bickmore *et al.* (1999) developed methods (including electrostatic attraction and adhesion based) to fix clay minerals to a substrate thus allowing imaging in aqueous suspensions by AFM. Further applications of AFM to environmental colloids have been reviewed by Maurice (1996). He describes the AFM as powerful tool to image environmental colloids and surfaces in air or immersed in water at sub-nanometre-scale resolution with examples of applications and limitations. Very recently a review has also been published relating the application of AFM to nanotechnology in food science (Yang *et al.* 2007).

From the above, it is clear that, using a combination of microscopic techniques, we can not only visualize nanoparticles but also generate useful data on the size, size distribution and other measurable properties (Baatz *et al.* 2006; Jose-Yacaman *et al.* 2001; Biberthaler *et al.* 2003; Rabinski and Thomas 2004; Chuklanov *et al.* 2006). However, it needs to be recognised that the image analysis of the microscope outputs is as crucial as imaging itself. Only small amounts of samples can be analysed by microscopic techniques and this has an impact on the statistical significance of the results. The average particle size is a number average, and size distribution obtained by image analysis depends on the number of particles measured. Since there are often fewer larger particles, it is important to count and measure enough particles to obtain good counting statistics on these size fractions. The same issues need to be considered when measuring ENPs in food or environmental samples in the presence

of high concentrations of natural nanomaterials. It may, therefore, be necessary to measure thousands of particles to generate reliable data. Therefore, it is essential to develop automation and image analysis procedures. Image contrast can have an influence on the visible size of the particles or light element particle coatings may be invisible, leading to controversial or incomparable results.

Chromatography and related techniques

Techniques based on or related to chromatography can be used for the separation of nanoparticles in samples. These techniques are rapid, sensitive (detector dependent) and non-destructive, so that samples are available for further analysis. Although some chromatographic tools allow a range of solvents to be used, samples usually cannot be run in their original media, which can cause sample alteration and sample solvent interaction. By attaching traditional analytical tools (*e.g.* ICP-MS, DLS) as detectors to size separation techniques, it is not only possible to quantify different nanoparticles in food, water, biota and soil, but also to characterise or elementally analyse them.

The best-known technique for size separation is size exclusion chromatography (SEC). A size exclusion column is packed with porous beads as the stationary phase. The pores of the column retain particles, depending on their size and shape. This method has been applied to the size characterisation of quantum dots, single-walled carbon nanotubes and polystyrene nanoparticles (*e.g.* Krueger *et al.* 2005; Ziegler *et al.* 2005; Huang *et al.* 2005). Size exclusion chromatography has good separation efficiency, but major disadvantages include possible interactions of the solute with the solid phase or the limited size separation range of the columns, which may not cover the size range of both the primary nanoparticles and their aggregates. Methods employed to overcome the problem of solid-phase interactions include the addition of capping agents to the mobile phase and the recycling of the analyte. SEC has been successfully combined with a range of detection techniques to not only monitor the size fractionation of the particles but also to characterise them. For example, Song *et al.* (2004) used voltammetric detection for gold nanoparticles separation and Helfrich *et al.* (2006) employed ICP-MS as a multi-element detection method, whereas Porsch

et al. (2005) worked with multi-angle laser light-scattering (MALLS) (Porsch *et al.* 2005).

Unlike SEC, in capillary electrophoresis (CE) there are no solid phase interactions. CE allows the separation of particles in different solutions based on the charge and size distribution of the components. However, as separation is not based on size alone, data interpretation is more complex. Also, mobile phase interactions cannot be excluded. Lin *et al.* (2007) used CE for the sizing of engineered Au and Au/Ag nanoparticles and Schmitt-Kopplin and Junkers (2003) have used CE in the characterisation of humic substances and other natural organic matter.

Hydrodynamic chromatography (HDC) separates particles based on their hydrodynamic radius. A HDC column is packed with non-porous beads building up flow channels in which particles are separated by flow velocity and the velocity gradient across the particle. Therefore, larger particles elute faster from the column than smaller ones (Mcgowan and Langhorst 1982). The non-porous beads considerably reduce the risk of solid-phase interactions compared to the porous packaging in a SEC column. Available HDC columns show size separation ranges from 5 to 1200 nm depending on the column length, whereas the size separation range of a SEC column is dominated by its pore size distribution. The wider particle size-separation range of HDC allows a whole range of nanoparticles to be sized in different media and is particularly helpful in allowing a better understanding of formation of aggregates. HDC has been connected to the most common UV-Vis detector for the size characterisation of (fluorescent) nanoparticles, colloidal suspensions and biomolecules (Williams *et al.* 2002; Chmela *et al.* 2002; Blom *et al.* 2003), but also to dynamic light scattering (DLS) for sizing separate lipid nanocapsules (Yegin and Lamprecht 2006). A major limitation of HDC is poor peak resolution.

A highly promising technique for the size separation of ENPs in complex natural samples is field-flow fractionation (FFF) (Giddings 1993; Beckett and Hart 1993; Schimpf *et al.* 2000). It is similar to chromatographic techniques, but separation is solely based on physical separation in an open channel without relying on a stationary phase. The particles are separated based on how they are affected by an applied field. The field controls the particle transport velocity by positioning them in

different average laminar flow vectors in a thin channel. The field can be a centrifugal force (sedimentation FFF) or a hydrodynamic flow perpendicular to the separation flow (flow FFF). FFF is able to fractionate particles in a range from 1 nm to 1 mm in Brownian mode.

FFF instruments can be coupled to online or offline detection and characterisation, which in addition to size distributions, allows analysis and visualisation of the fractionated samples by electron microscopy (Baalousha *et al.* 2005a). FFF can also be coupled to a range of sensitive and multi-element techniques, such as multi-angle laser light-scattering (MALLS) and ICP-MS (Hassellöv *et al.* 1999b; von der Kammer *et al.* 2005a). FFF coupling techniques have been successfully applied in geochemistry and natural colloid research as well as studies into the behaviour of engineered nanoparticles. Applications range from colloids in fresh and marine water to size separation of soil suspensions (Ranville *et al.* 1999; Hassellöv *et al.* 1999a; Hassellöv *et al.* 1999b; Chen and Beckett 2001; Lyven *et al.* 2003; Siepmann *et al.* 2004; von der Kammer *et al.* 2004; von der Kammer *et al.* 2005a; Stolpe *et al.* 2005; Baalousha *et al.* 2005a; Graff and Frazier 2006; Lead and Wilkinson 2006; Gimbert *et al.* 2006; Peng *et al.* 2006; Baalousha *et al.* 2006a; Baalousha *et al.* 2006b; Baalousha and Lead 2007). Also, single walled carbon nanotubes have been length-separated by dielectrophoresis FFF (Peng *et al.* 2006) and many engineered nanoparticles, such as SiO_2, metals, metal oxides, carbon black, etc. have been analysed by FFF (Schimpf *et al.* 2000).

The limitations of FFF techniques are membrane or accumulation wall interactions, the continuous re-equilibration in the channel (for trace constituent studies) and the need (in some circumstances) of preconcentration, additional concentration of sample during equilibration and an increasing possibility of aggregation in the channel (Beckett and Hart 1993; Hassellöv *et al.* 2007).

In theory, any aqueous or non-aqueous phase of any ionic strength and a pH between 2 and 11 can be used as a carrier. This gives versatility in terms of selecting the carrier composition to favour colloidal stability, thus minimizing wall and membrane interactions and particle-particle interactions.

Stegeman *et al.* (1994) compared the resolving power and separation time in thermal field-flow fractionation (TFFF), hydrodynamic chromatography and size exclusion

chromatography for the size separation of polymers, and concluded that TFFF theoretically has the best separation potential due to high selectivity, but this may not be exploitable in practice owing to the technical requirements. On the other hand, SEC was found to be the fastest method for low molecular masses (Stegeman *et al.* 1994). In general, FFF and HDC have a wider dynamic size range than SEC, while SEC has higher separation efficiency (less peak broadening). SEC also suffers from more sample perturbations than FFF or HDC.

Centrifugation and filtration techniques

Centrifugation and filtration techniques are well-established tools for the preparative, size fractionation of samples. These are low-cost, high speed and high volume techniques. Ultracentrifugation (UC), for example, is a centrifuge system capable of very high spinning speeds for accelerations up to 1,000,000 g. There are two different types of ultracentrifugation: analytical and preparative UC. In an analytical ultracentrifuge (ANUC), a sample can be monitored in real time through an optical detection system using ultraviolet light absorption and/or interference optical refractive index sensitive systems. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field, and is valuable for sedimentation velocity and sedimentation equilibrium experiments (gross shape of macromolecules, conformational changes in macromolecules and size distribution). Preparative ultracentrifugation has been used for pelleting of fine particulate fractions, for gradient separations (Bootz *et al.* 2004) and for harvesting aquatic colloids and nanoparticles on TEM and AFM substrates (Mavrocordatos *et al.* 2007; Balnois *et al.* 2007).

Traditional membrane filtration allows the fractionation of particle sizes between 0.2 and 1 mm (Lead and Wilkinson 2006). Comparative data obtained for soil suspensions, for filtration and sedimentation FFF indicates that membrane filtration can both over- and under-estimate smaller size fractions due to clogging as well as electrostatic interactions (Gimbert *et al.* 2005). Microfiltration with pore sizes > 0.1 µm is a simple and common method, although exhibiting many artefacts caused by, for example, filter-cake formation and concentration polarisation

(Morrison and Benoit 2001). Ultrafiltration is applicable for large sample volumes; however, with decreasing pore sizes, common filtration artefacts are even more likely. For the separation of nanoparticles and ions, nanofiltration with pore sizes of 0.5 or 1 nm can be used.

Cross flow filtration (CFF) or tangential filtration recirculates the samples and, therefore, reduces clogging, concentration polarisation and other artefacts caused by traditional dead-end filtration (Lead and Wilkinson 2006). It has become the standard method for separating colloids and particles and its efficacy has been evaluated against AFM by Liu and Lead (2006). The method has been applied to fluorescence investigations of colloidal organic matter and dissolved organic matter in lake and river water (Liu *et al.* 2007) as well as in seawater (Guo *et al.* 2000). Electrically assisted cross-flow filtration has also been used for the separation of nanoparticles (Sung *et al.* 2007). Doucet *et al.* (2004) evaluated cross-flow ultrafiltration (CFUF) for the size fractionation of freshwater colloids and particles (1 nm – 1 mm) by AFM and SEM, and concluded that CFUF is not fully quantitative and separation is not always based on size alone. Amounts of large colloids might be overestimated and fractionation is not always consistent with the nominal pore size of the membranes. These conclusions have to be treated with some caution as the validation techniques used (*i.e.* AFM and SEM) also have their limitations (Doucet *et al.* 2004).

Spectroscopic and related techniques

A wide range of spectroscopic methods is available for nanoparticle analysis and characterisation. Scattering techniques useful for nanoparticle characterisation include light scattering methods, such as static (SLS) and dynamic light scattering (DLS), or neutron scattering, such as small-angle neutron scattering (SANS).

DLS or photon correlation spectroscopy (PCS) is particularly useful for sizing nanoparticles and determining their state of aggregation in suspensions. DLS provides fast *in situ* and real-time sizing (Ledin *et al.* 1994), but also has considerable limitations. For example, interferences can be caused by a range of possible artefact sources, such as dust particles, which will influence the scattering intensity compared to smaller particles and, therefore, on the sizing result. Also, data obtained from samples containing particles with heterogeneous size distributions are

difficult to interpret. DLS is solely quantitative and unless the sample content is known or pure, size fractions cannot be related to particles of a specific composition (*e.g.* Bootz *et al.* 2004).

Static light scattering, also known as multi-angle (laser) light-scattering (MAL(L)S), gives information on particle structure and, in combination with dynamic light-scattering or FFF, particle shape can be determined.

SANS can be used on solid or liquid samples. For example, Diallo *et al.* (2005) have applied SANS for the characterisation of Suwannee River fulvic acid aggregates in aqueous solutions.

Small angle X-ray scattering (SAXS) is an analytical X-ray application technique for investigating the structural characterisation of solid and fluid materials in the nanometre range. Monodisperse and polydisperse systems can be studied. In monodisperse systems, size, shape and structure determination is possible, whereas, in polydisperse systems, only the size distribution can be calculated.

Laser-induced breakdown detection (LIBD) is a laser-based technique featuring extremely low detection limits, which is capable of analysing the size and concentration of colloids, depending on the measured breakdown probability (BP). LIBD is, therefore, a highly promising tool for nanoparticle characterisation, although it cannot distinguish between different types of particles and requires particle-specific size calibration (Bundschuh *et al.* 2001a; Bundschuh *et al.* 2001b).

Other laser-based techniques include Raman spectroscopy and laser-induced fluorescence (LIF). Instruments are now available combining these techniques, allowing the atomic, molecular and structural characterisation of a specimen, as well as a better understanding of physical properties.

UV-Vis and infrared spectroscopy offer the possibility of characterising nanoparticles, especially quantum dots and organic-based nanoparticles, such as fullerenes and carbon nanotubes. Fourier transformation infrared (FTIR) and UV-Vis spectroscopy have been used to compare aqueous colloidal suspensions of C60 (Andrievsky *et al.* 2002). Pesika *et al.* (2003) also used UV spectroscopy to study the relationship between absorbance spectra and particle-size distributions for quantum-sized nanocrystals.

Nuclear magnetic resonance (NMR) is a powerful technique providing information on the dynamics and three-dimensional structure of a solid compound or a suspension. Carter *et al.* (2005) characterised air- and water-stable silica nanoparticles by NMR. Diffusion NMR spectroscopy has also been used for the characterisation of the size and interactions of colloidal matter (Valentini *et al.* 2004; Carter *et al.* 2005). Lead *et al.* (2000a) used pulsed field gradient NMR to measure the diffusion coefficients of fulvic acids.

X-ray spectroscopy comprises X-ray photoelectron (XPS), X-ray fluorescence (XRF) as well as X-ray absorption spectroscopy (XAS) and X-ray diffraction (XRD). XPS is highly surface-specific due to the short range of the photoelectrons that are excited from the solid sample and, therefore, XPS could be useful to characterise nanoparticle surfaces and coatings. X-ray diffraction is non-destructive and can reveal information about the crystallographic structure or elemental composition of natural and manufactured materials. Nurmi *et al.* (2005) used this technique, as well as XPS, for the characterisation of zero-valent Fe nanoparticles for use in remediation. X-ray fluorescence (XRF) spectroscopy is also non-destructive and can be used to identify and determine the concentrations of elements present in solid, powdered or liquid samples. XRF can be subdivided into wavelength separation (WDXRF) and energy dispersive XRF (EDXRF).

X-ray absorption (XAS) and emission spectroscopy is used in chemistry and material sciences to determine elemental composition and chemical bonding.

Other potentially suitable spectroscopic techniques for nanoparticle characterisation include electron paramagnetic resonance (EPR), Mössbauer, Auger electron (AES) and 3D fluorescence excitation-emission matrix spectroscopy (EEM). Mössbauer spectroscopy provides information about chemical, physical and magnetic properties by analysing the resonant absorption of characteristic energy gamma-rays, known as the Mössbauer effect. Liu *et al.* (2007) and Lead *et al.* (2006) applied 3D fluorescence excitation-emission matrix (EEM) spectrophotometry for the fluorescence investigation of colloidal organic matter and dissolved organic matter in lake and river water. EPR spectroscopy can be applied for particle surface reactivity analysis, and is a sensitive, specific method for studying organic and inorganic radicals formed in chemical reactions or the reactions themselves, similar to NMR.

Auger electron spectroscopy is also commonly used in the surface characterisation of nanostructures. Quantitative bulk analysis by AES has been described by Powell and Seah (1980).

Mass spectrometry

Mass spectrometers consist of an ion source, a mass analyser and a detector system. Two ionization techniques often used with liquid and solid biological samples include electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).

Inductively coupled plasma (ICP) sources are mainly used for metal analysis. Mass analysers (*e.g.* ion trap, quadrupole or time-of-flight) cover different mass-to-charge ranges, differ in mass accuracy and achievable resolution. Most of the available analysers are compatible with electrospray ionization, whereas MALDI is not usually coupled to a quadrupole analyser.

Mass spectrometry (MS) approaches, such as MALDI, laser-induced fluorescence (LIF) or ion trap (IT) mass spectrometry, have been applied for the analysis of fluorescently labelled nanoparticles (Peng *et al.* 2003; Cai *et al.* 2003).

In the case of ICP-MS, samples cannot only be injected directly into the ion source but via a combined technique, such as HPLC. An increasingly popular combination in this respect is FFF-ICP-MS, which allows the size separation of the sample with quantitative and elemental analysis of the obtained size fractions. This development is highly promising for nanoparticle analysis, as particles can be simultaneously sized and analysed in their original environment (Ranville *et al.* 1999; Lyven *et al.* 2003; von der Kammer *et al.* 2004; Bolea *et al.* 2006; Baalousha *et al.* 2006a).

Whereas conventional mass spectrometry (MS) is applicable for identifying unknown compounds and their mass concentrations, as well as their isotopic composition, single particle mass spectrometry (SPMS) has also the ability to size single particles. MS techniques have also been used in aerosol characterisation, including aerosol time-of-flight mass spectrometer (ATOF-MS). An ATOF-MS consists of an aerosol introduction interface; a light-scattering region for sizing and a TOF–MS. Suess and Prather (1999) published a review on the topic of mass spectrometry of aerosols, describing tools for offline MS of aerosols, such as

LAMMS, SIMS and ICP-MS, tools for online MS, such as surface/thermal ionization MS (SIMP, DIMS, CAART, PAMS), and laser desorption/ionization MS (ATOFMS, PALMS, RSMS, LAMPAS). More applied examples are described by Janzen *et al.* (2002), who compared the sizing of nanoparticles with SPMS and TEM. Lee *et al.* (2005) used SPMS to characterise the size and composition of polydisperse aerosol nanoparticles. They estimated particle size with a laser ablation/ionization time-of-flight single-particle mass spectrometer and validated their results by differential mobility analysis (DMA). *In situ* characterisation of size and elemental composition of individual aerosol particles in real time was performed by Prather *et al.* (1994) with the help of an ATOF-MS. For the sizing and analysis of aerosol nanoparticles, a DMA has also been coupled to an ICP-MS (Okada *et al.* 2002).

Other techniques

Particle counters for number concentrations. The electrical sensing zone method counts and sizes particles by detecting changes in electrical conductance as particles suspended in a weak electrolyte solution are drawn through a small aperture. The technique has been successfully applied to the size and surface charge characterisation of nanoparticles using a carbon nanotube-based Coulter counter (Ito *et al.* 2003). Condensation particle counter (CPC) measurements can also provide data on the number and concentration of individual particles by growing the particles through a condensing process using various operating liquids, such as alcohol and water.

DMA for sizing aerosols. A differential mobility analyser (DMA) can be used to determine the size distribution of sub-micrometer aerosol particles. Particles are firstly charged and then their electrical mobility is measured as a function of their charge and size. After sizing, the particles are still suspended in air and are ready for further analysis (McMurry *et al.* 1996; Weber *et al.* 1996; Okada *et al.* 2002).

SMPS for sizing and number concentration determination. A scanning mobility particle sizer (SMPS) consists of a DMA and a CPC. First, particles are separated by their electrical mobility in the DMA; then, the size fractionations enter a CPC, which determines the particle concentration at that size.

BET method for surface area determination. The very common Brunauer–Emmett–Teller (BET) method enables the determination of the specific surface area of solids and, thus, nanoparticles by gas adsorption (Brunauer *et al.* 1938).

Thermogravimetry and differential thermo analysis (TG-DTA). DTA can be applied for phase changes and other thermal processes, such as the determination of melting point. In combination, TG-DTA is useful for investigating the thermal stability and decomposition, dehydration oxidation, as well as the determination of volatile content and other compositional analysis. Thermogravimetry in combination with a mass spectrometer can be used for surface analysis. Surface molecules are removed by heating and afterwards analysed by MS.

Electrophoretic mobility and the zeta potential. Electrophoresis is used for studying properties of dispersed particles, in particular, for measuring the zeta potential. The zeta potential is a measure of the overall charge a particle acquires in a specific medium and gives an indication of the potential stability of a colloidal system. If all the particles have a large negative or positive zeta potential, they will repel each other, which leads to higher stability than if the particle charge is nearly neutral. The zeta potential is a measure of the net charge and there may be significant charge heterogeneities that can still lead to aggregation, even though the net zeta potential suggests otherwise. Information about the aggregation state of a nanoparticle dispersion is highly valuable for nanoparticle fate and behaviour studies. As an example, the electrophoretic mobility of silica spheres suspended in water at different concentrations and salinities has been studied by Reiber *et al.* (2007).

Nanomaterial analysis in food and biological samples

As previously discussed, when measuring nanoparticles in different media, it is not only necessary to generate data on concentrations but information will also be required on the size distribution and properties of the particles. No single technique can provide all this information, so a range of analytical techniques is required. Moreover, while a range of methods has been shown to be applicable to the analysis of nanoparticles, the current methods do not fulfil all data requirements.

As shown in the previous section, many analytical tools are theoretically suitable for the characterisation of nanoparticles, ranging from electron microscopy to dynamic light scattering to field flow fractionation techniques, but only a few of these are applicable to the analysis of more complex samples. Requirements for analysis of engineered nanoparticles in natural and food related samples will differ greatly from their analysis in pure or neutral media (*e.g.* air, distilled water). In complex media, it is essential to analyse samples of diverse elemental compositions and samples containing more than one type of nanoparticle. Many techniques are destructive or, if not, application of some sample preparation methods can lead to artefacts. In addition, natural samples will be heterodispersed and, for measuring size distributions, instruments providing a wide size-separation range from, ideally, 1 nm to up to several mm, are needed. There are many methods available for the sizing of particles, but very few, if any, is applicable to the entire size range. In the next section, some of these challenges are discussed in more detail.

Bulk versus single particle analysis

One problem with some methods, as discussed previously, is their application range. Existing techniques have to be divided between tools suitable for analysing individual particles (depending on particle size) or the bulk material. Classic composition and mass-based tools are readily applicable for the bulk material; however, elemental analysis of single particles in a dilute environment has only recently become available (*e.g.* aerosol mass spectrometry). Whereas, standard tools for elemental composition and mass concentration are restricted by their limit of detection (LOD), techniques capable of characterising individual particles face

spatial limitations. Especially, particle sizing techniques are restricted by their size separation range. Figure 2.2 illustrates the size range of selected methods for particle sizing.

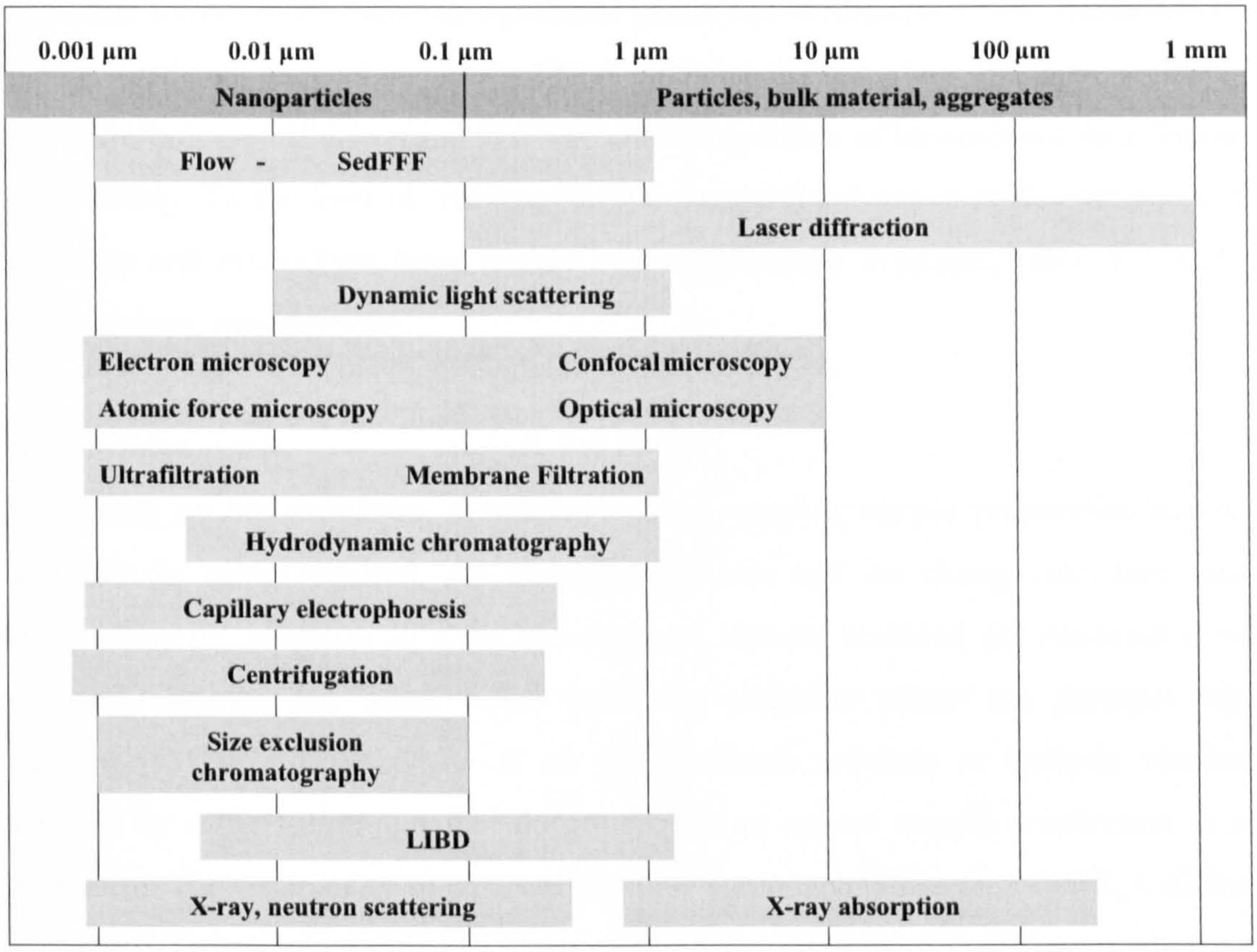


Figure 2.2. Sizing methods and their size range for nanoparticle measurement. Adapted from Lead and Wilkinson (2006) and Gimbert *et al.* (2007b).

Sizing artefacts and the lack of reference materials

The limitations of each analytical method for nanoparticle characterisation can lead to inconsistent results and, therefore, to inaccurate predictions of material properties and structure (Carter *et al.* 2005). For example, it is still almost impossible to determine the absolute size of particles. Correct size measurements are difficult, which often leads to artefacts, depending on the applied tool and the medium the particles are analysed in. For example, organic coatings that are not visible in the electron microscope (due to light elements, such as carbon) can lead to errors in sizing, especially when compared to sizing tools that measure the hydrodynamic

radius of particles, such as FFF or DLS. It has been reported that the average size and size distribution of nanoparticles can significantly vary when comparing results from different techniques, such as electron microscopy, dynamic light scattering, CFF or ultracentrifugation (Bootz *et al.* 2004). The lack of consistent reference materials and standards further exacerbates this problem (Lead and Wilkinson 2006). Nanoparticle sizing standards, as well as standardized methods for sampling and measurement, are, therefore, urgently required to overcome the problem of inconsistent data (Borm *et al.* 2006). To the best of our knowledge, standardized nanoparticles are not yet available and researchers have to rely on commercially available, often not well-characterised, nanoparticles.

Sample preparation

Depending on the technique, to analyse natural samples, sample preparation and/or digestion is often required. As nanoparticles can and do change structure and composition in response to their environment, results obtained for pre-treated or digested samples can often differ from the situation where the particles are characterised *in situ* (Burleson *et al.* 2004). These artefacts in analysis can be avoided by using techniques that do not require or reduce sample preparation to a minimum. The complexity of data obtained for some techniques (*e.g.* NMR, CE) for samples in their original state can make analysis and interpretation difficult.

If sample preparation cannot be avoided, a careful record of sampling and preparation steps is essential to track artefacts. The nature of nanoparticles can also change over time; for example, aggregation can increase or decrease and particles could dissolve. A lot of effort has been put into the development of sample preparation methods that improve the conservation of the original state of the sample. Especially in the field of microscopy, advances have been made in sample preparation ranging from gel-trapping techniques for imaging emulsions under the SEM (Paunov *et al.* 2007) to high-pressure freezing and freeze-drying for imaging biological specimen under the TEM (Lonsdale *et al.* 1999; Bootz *et al.* 2004). Fixation methods for imaging clay minerals and particles in aqueous solutions under the AFM have also been developed (Bickmore *et al.* 1999).

Natural versus engineered nanoparticles

At the moment, it is very difficult to distinguish between particles of engineered origin and particles from a natural or other source (Burleson *et al.* 2004). A way has to be found to differentiate between natural occurring and engineered nanoparticles. As the number of engineered nanoparticles actually reaching the environment or their bioavailability is unknown, this will allow concentrations in consumer products and the environment to be determined. Therefore, selective detection methods need to be developed. Another solution to this problem could be nanomaterial labelling, with suggestions ranging from fluorescent- and radioactive-labelling for carbon-based nanoparticles to isotopic enrichment or depletion of metal-based nanoparticles. Also, special particle coatings or entrapment of rare elements in nanotubes or fullerenes could be used to enable the detection of these distinctive chemical characteristics after an experimental study. Gulson and Wong (2006) reviewed the possibilities of isotopic labelling and tracking of metal and metal oxide nanoparticles for nanotechnology research. Isotopic labelling of carbon nanotubes and fullerenes has already been performed; for example, ^{13}C isotope carbon nanotubes are available and ^{14}C-C60s have been synthesized, with subsequent uptake and toxicity studies (Scrivens *et al.* 1994b; Bullard-Dillard *et al.* 1996).

Conclusions and recommendations for future work

Analytical methods are required to reliably detect and characterise nanoparticles and their properties in matrices to which humans and ecosystems are exposed, including air, soil and water as well as food and consumer products. These methods must also be applicable for nanoparticle characterisation in toxicological and ecotoxicological testing; only then can an appropriate risk assessment be performed and nanoparticle properties of risk identified and regulated or used in standard testing (Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR) 2005).

These techniques have to be able (a) to deal with heterogeneous samples, (b) minimize sample alteration to avoid artefacts and (c) provide as much information as possible, because most characterisation techniques are destructive and, therefore,

samples often cannot be analysed twice or by more than one technique. An ideal analytical instrument should allow simultaneous determination of all physicochemical properties of a nanoparticle and, as many nanoparticles are transient in nature, obtain them by real-time sampling (Prather *et al.* 1994). While a wide range of tools is available, the existing tools do not fulfil all desirable criteria and have limitations when considering their application for food and natural samples. Therefore, until new tools have been developed, existing tools have to be used and combined in such a way that data can be validated. Analysis of the unperturbed sample or further analysis of the size fractionations is preferred. Complementary analytical tools should be applied and care taken with sample preparation.

As this review demonstrates, promising developments have been made in nanoparticle analysis; however, further advances are essential to overcome these deficiencies. Especially, *in situ* analysis as well as routine and reliable techniques to improve size determination, size distribution of particles and other nanoparticle properties are important.

Nanotoxicology and nanoecotoxicology are still in their infancy and risk assessments are practically nonexistent, especially in the food sector. Therefore, progress in nanoparticle testing (in vivo and in vitro) is urgently needed to guarantee consumer safety, including the development of standard testing materials and testing guidelines. In addition to toxicity studies, various uptake paths have to be studied, including dermal, oral and intestinal, as well as nanoparticle accumulation and potential long-term effects. Other effects of nanoparticle uptake could be the interaction with other (toxic) substances and their mobilisation or dislocation, not only in the human body, but also in consumer products. The environmental fate, behaviour and bioavailability of nanoparticles are unknown and, thereby, their potential impact on food webs and persistence. Their effect on other substances also needs examination; for example, whether contaminant transport in the environment could be facilitated through adsorption to nanoparticles, whether nanoparticles enhance contaminant uptake or have a negative impact on bacteria useful for natural remediation. Furthermore, data on environmental and exposure concentrations are unavailable. Developments in the above-mentioned analytical fields will be crucial to further our knowledge of nanoparticle and related issues.

In the work described in the following two chapters, two of the approaches identified in this review as having potential to provide useful data on the characteristics of engineered nanoparticles in aquatic systems, are explored in more detail. Chapter 3 describes an evaluation of the WetSEM^{TM} method using metal-based ENPs and a range of complex aquatic matrices. In chapter 4, an HDC-ICP-MS method is developed and evaluated for detecting and characterising metal-based ENPs in aqueous samples of varying complexity.

Chapter 3

Imaging of engineered nanoparticles under fully liquid conditions in environmental matrices

Introduction

Electron and atomic force microscopy (EM and AFM) have proven to be powerful tools for the imaging and characterisation of nanoparticles. The conventional application of EMs for environmental samples, *e.g.* nanoparticles in natural waters, is, however, still a challenge (see chapter 2). For example, in scanning and transmission electron microscopy (SEM, TEM), due to the vacuum conditions in the sample chamber, sample preparation such as coating, drying, staining, freezing and embedding is essential and therefore only perturbed samples can be imaged. This can lead to imaging artefacts. Environmental SEM (ESEM) is a possibility to overcome the vacuum conditions in the SEM sample chamber and allows imaging of hydrated samples (Bogner *et al.* 2005). However, imaging of fully unperturbed samples in an ESEM is not yet possible (Redwood *et al.* 2005) as standard pressure conditions and also imaging under fully wet conditions cannot be achieved, as at 100 % relative humidity the hydrated samples are fully covered by a layer of free water, which makes particle visualisation impossible (Doucet *et al.* 2005a).

One possible approach to address the limitations of AFM, TEM, SEM and ESEM is to employ WetSEM™ technology. WetSEM™ employs stainless steel capsules designed by Quantomix™ (Quantomix, Israel, Internet 2008). The capsules are equipped with an electron transparent membrane so that wet samples can be placed into the capsules and imaged in a standard SEM (Barshack *et al.* 2004a; Thiberge *et*

al. 2004b; Joy and Joy 2006). The approach has been applied to samples in liquid *e.g.* un-manipulated biological samples such as cells and tissues (Thiberge *et al.* 2004a; Barshack *et al.* 2004b). It may however be a very useful technique for imaging ENPs in environmental matrices.

The aim of the work described in this chapter was therefore to explore the potential for WetSEM^{TM} to be used as an imaging technique for characterising the fate and behaviour of ENPs in aquatic systems. Experiments were performed on a range of metal and metal oxide nanoparticles (Au, TiO_2, ZnO and Fe_2O_3) and a range of aqueous media (distilled water, lake water and a soil suspension). Results obtained using WetSEM^{TM} were compared with conventional SEM, TEM/EDS and AFM. As similar results were found for the conventional methods, only results from SEM are presented in the following sections.

Methods

Preparation of metal and metal oxide samples in distilled water, lake water and soil suspension

Gold nanoparticles in water dispersion (average size: 50 nm) were purchased from BBInternational, UK and metal oxide nanoparticles in powder form in size ranges of 50-70 nm for ZnO NPs, 5-10 nm for TiO_2 NPs and 20-25 nm for Fe_2O_3 NPs as stated by the manufacturer from Sigma Aldrich, UK. Samples of TiO_2, ZnO and Fe_2O_3 as well as mixtures of these metal oxides were prepared in both distilled and lake water (lake water sampled from a lake on the grounds of the Central Science Laboratory, UK and distilled water by Millipore, UK) and kept refrigerated. Initial particle concentrations dispersed in the sample liquid were ~ 5 mg/L. Au nanoparticles (~ 10 mg/L) were added to a well-characterised soil suspension (soil in distilled water, soil: Wedgenock UK, OS map reference: SP26826645, 41.5 % clay, 5.58 % organic matter, 4.9 pH $CaCl_2$, < 1 % $CaCO_3$ and 22.3 % CEC).

Sample preparation and imaging of metal-based nanoparticles by conventional SEM

Samples of TiO_2, ZnO and Fe_2O_3 as well as mixtures of these metal oxides for SEM imaging were prepared by applying 15 µL (corresponding volume to WetSEM™ capsules) to Aluminum stubs with Carbon discs (Agar Scientific, UK) or Silicon, and allowed to dry. Imaging took place in a FEI Sirion™ FEGSEM under standard working conditions (HR mode, acceleration voltage, spot size, magnification, detector mode and working distance are stated on figures or in figure legends). Additionally, in-situ qualitative elemental analysis of the samples was performed with an Oxford Instruments INCA EDS x-ray analysis using the Sirion in EDX mode.

WetSEM™ imaging

The ability of imaging and chemically analysing metal oxide nanoparticles under fully liquid conditions was achieved by using capsules developed for WetSEM™ by Quantomix (Quantomix, Isreal; Thiberge *et al.* 2004a). These QX capsules comprise an electron transparent membrane enabling the imaging and elemental analysis of liquid samples (Thiberge *et al.* 2004b) in a conventional SEM/EDS.

Two different capsule types were applied: The QX-102 capsule (figure 3.1) is applicable to liquid samples and particles that can be adhered or are close to the capsule membrane (capsule volume 15 µL). The QX-302 is suitable for imaging thick or solid, non-adherent samples such as tissue and plants in a wet environment with a maximum diameter of 3 mm and sample thickness up to 1 mm.

Imaging of all liquid samples (described below) took place in the above-described FEI Sirion™ FEGSEM instrument. In order to reduce electron beam damage to the thin membrane of the capsules it was best to choose high incident beam energies of 20-30 keV rather than lower keV, and to minimise excessive dwell times of the beam on areas of the membrane such as when imaging at high magnification or when using spot mode EDS. Through-the-lens detection (TLD) was found to give higher contrast in the image than standard secondary electron detection because of smaller collection angle that the TLD has, which reduces the collection of secondary electrons generated within the membrane. Applied working conditions are shown in the respective figures.

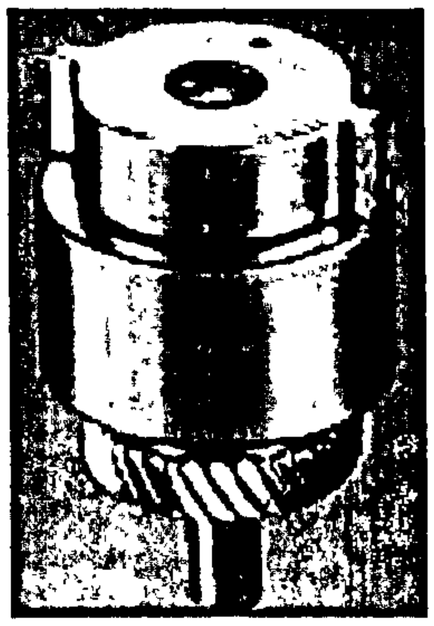


Figure 3.1. QX-102 capsule.

WetSEM™ imaging of metal-based nanoparticles in distilled water. First, the QuantomiX™ QX-102 capsule was applied to image metal-based nanoparticles in distilled water. 15 µL of the respective samples (samples of TiO_2, ZnO and Fe_2O_3 as well as mixtures of these metal oxides in distilled water) were pipetted into the liquid dish of the QX-102 capsules in accordance with the supplier instructions one to seven days prior to imaging. No further sample preparation was performed. The capsules were kept dark and refrigerated until analysis.

WetSEM™ imaging of metal-based nanoparticles in lake water. After imaging of metal oxide nanoparticles in pure media, the performance of the capsules was assessed using more complex media. Thus, samples of TiO_2, ZnO and Fe_2O_3 as well as mixtures of these metal oxides in lake water were applied to QX-102 capsules and imaged as described above.

Capsule membrane coating for improved WetSEM™ imaging of metal-based nanoparticles in lake water. As the beam can penetrate ~ 2-3 µm into the sample, objects have to be close to the membrane to be imaged. For better adhesion of particles to the capsule membrane and therefore improved imaging of lake water samples, membrane coating prior to analysis was tested. QX-102 capsules were treated with three different membrane coatings to improve adherence of negatively and positively charged as well as neutral particles respectively according to the coating protocols available on the manufacturer's website (Quantomix 2008).

To improve attachment of neutral particles to the capsule membrane, the membrane was coated with gelatine a day before usage. For this, 0.1 % w/v Gelatine was dissolved in distilled water at 37 °C until no lumps were visible. 15 µL were then applied to the liquid dish and incubated for one hour. The solution was removed and the dish rinsed twice with phosphate buffered saline (PBS). Then 15 µL of the metal oxide nanoparticle spiked lake water sample were applied to the pre-treated liquid dish and incubated for one hour at room temperature prior to imaging.

To achieve attachment of negatively charged particles to the capsule membrane, the membrane was coated with poly-l-lysine. For this, 0.1 % w/v poly-l-lysine in distilled water were applied to the liquid dish and incubated overnight at room temperature. The solution was removed and the dish rinsed twice with distilled water. Then 15 µL of the metal oxide nanoparticle spiked lake water sample were applied to the pre-treated liquid dish and incubated for one hour at room temperature prior to imaging.

To achieve attachment of positive charged particles, capsule membranes can be coated with poly (sodium-4-styrenesulfonate) (PSS). For this 15 µL of a PSS solution of 0.3 % w/v in distilled water have been applied to the capsule liquid dish and incubated overnight at room temperature. The solution was removed and the dish rinsed twice with distilled water. Then 15 µL of the metal oxide nanoparticle spiked lake water sample were applied to the pre-treated liquid dishes and incubated for one hour at room temperature prior to imaging.

WetSEM™ imaging of metal-based nanoparticles in a soil suspension. Sample complexity was then further increased by spiking gold nanoparticles to a soil suspension. For this a different type of capsule, the QX-302, was used. A Gold nanoparticle - soil suspension was applied to a spacer (Whatman® 3 mm CHR, diameter: 3 mm, thickness 0.3 mm) and then to the capsule. Attachment of the sample to the capsule membrane is mechanically achieved by adding more spacers (depending on the thickness of the sample) and by the sealing stub, which contains a plunger that, once the capsule is closed, pushes the sample towards the membrane. Samples were imaged by SEM/EDS, using the methods described above, and a

through-the-lens detector (TLD) was used. Applied working conditions are shown in the respective figures.

Image analysis for all images was performed using the public domain Java image-processing program ImageJ.

Results and discussion

Using WetSEM^{TM} we achieved the visualisation of metal-based ENPs under fully wet conditions minimising sample alteration and therefore imaging artefacts. The approach taken was to sequentially increase the complexity of the samples starting with metal-based nanoparticles in distilled water, then lake water and a soil suspension. Also capsule membrane coating approaches were tested to improve imaging. Results were compared to conventional scanning electron microscopy images. As the primary aim of the study was to develop a method for imaging engineered nanoparticles in liquids, spiking concentrations for the study nanoparticles were selected to ensure that the particles could be easily detected by the method. The concentrations used were therefore around two orders of magnitude higher than predicted environmental concentrations for engineered nanoparticles in use today (Boxall *et al.*, 2007). It is recognised that in the future, to make the method applicable to 'real' environmental situations, work will need to focus on lowering the detection limit of the method and/or developing approaches to concentrate samples prior to imaging.

WetSEM^{TM} imaging of metal-based nanoparticles in distilled water

Imaging under fully liquid conditions with the help of QX-102 capsules was achieved for Fe_2O_3 and TiO_2 in distilled water. ZnO nanoparticles could not be observed in these samples. This could be due to the fact that ZnO did not attach or was not close enough (> several microns) to the capsule membrane and thus could not be detected. Alternatively, the ZnO particles may have dissolved, making visualisation impossible. This is supported by recent studies that demonstrated that

ZnO nanoparticles dissolve rapidly in water (with a $Ca(NO_3)_2$ concentration 0.01 M and a pH of 7.5) up to an equilibrium concentration of around 16 mg of Zn per L (equivalent to 24 mg/L ZnO) after 72 h (Franklin *et al.*, 2007).

In figure 3.2 SEM and WetSEM™ images of TiO_2 in distilled water are shown. WetSEM™ was performed without any sample preparation and therefore alteration. Thus, images reveal a lower density of particles and aggregation than conventional SEM images and drying artefacts were avoided. However, particle sizes seemed to be comparable between SEM and WetSEM™ and show aggregate sizes of mostly > 1 µm. Using EDS, elemental characterisation of the particle clusters could be achieved (see EDS spectrograms, figure 3.2).

It has to be noted that particles are not necessarily fixed in the capsules and may be moving within the liquid phase. If this movement is on the same time scale as the image acquisition time there may be some loss of sharpness of the particles in the image (figure 3.2c). Additionally, particles further from the membrane will be imaged with slightly poorer spatial resolution than particles close to the membrane due to the increased electron scattering of the beam the further the beam travels in the matrix.

It was generally found that particles tended to move and drift away from the electron beam. Therefore, imaging and EDS analysis was rather challenging. Also, as the capsule membrane was sensitive to radiation damage, imaging time was limited and therefore objects of interest had to be found quickly and adjustment of imaging conditions *e.g.* focus and astigmatism correction as well as EDS analysis has to be done quickly.

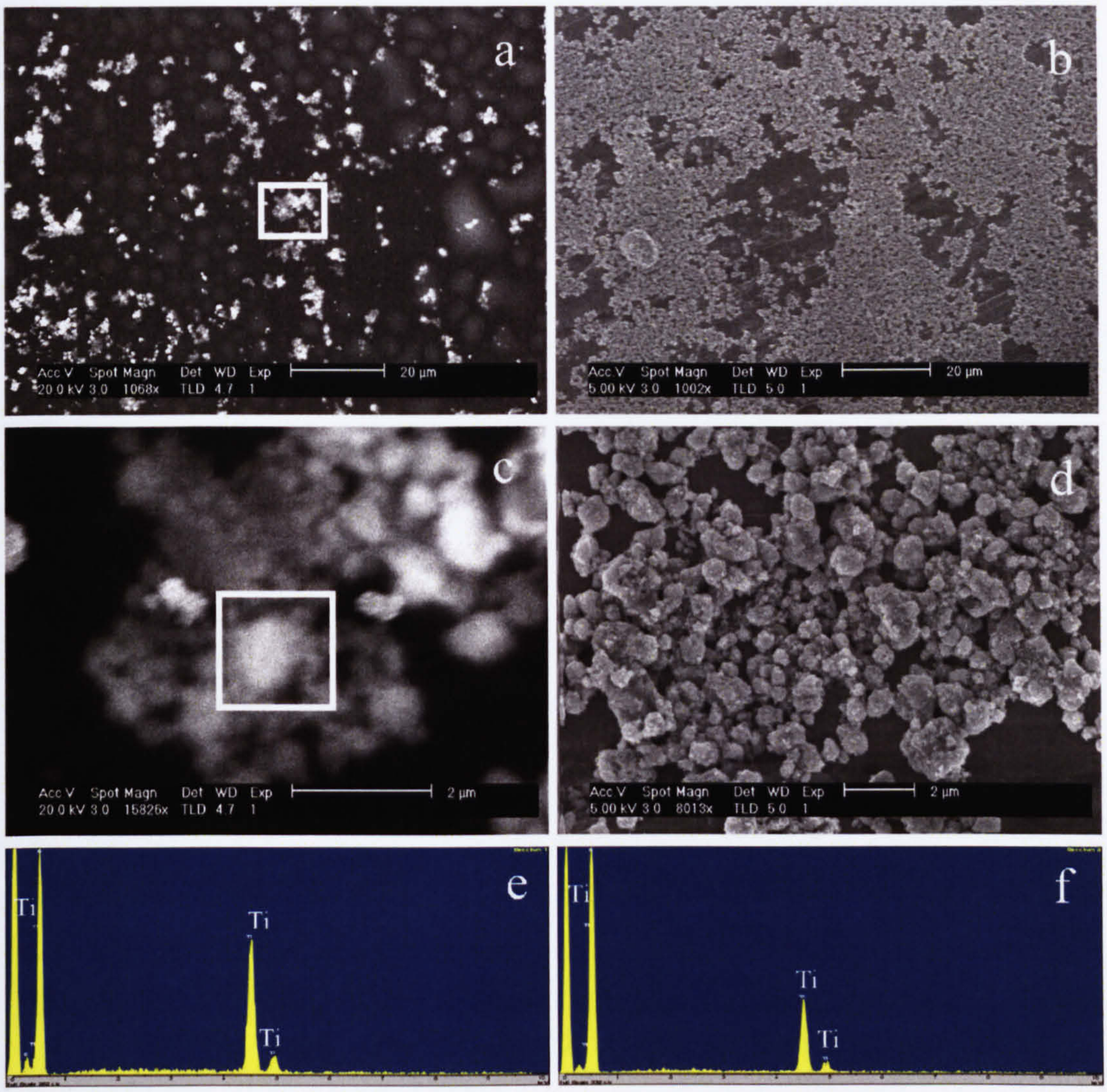


Figure 3.2. TiO_2 in distilled water imaged by WetSEM™ (a, c) and conventional SEM (b, d). Dried sample as imaged by conventional SEM indicate a higher state of particle aggregation compared to the image under fully wet conditions. Image 3.2c shows difficulties in image sharpness probably due to moving particles in water. Scale bars indicate 20 µm in top row and 2 µm bottom row. X-ray spectra are acquired for the areas indicated by the white square boxes. EDS spectrograms confirmed the element titanium as major component of the particles and are shown for images a (e) and c (f).

WetSEM™ imaging of metal-based nanoparticles in lake water

To achieve visualisation of nanoparticles under liquid conditions in natural environments, TiO_2 Fe_2O_3 and ZnO nanoparticles were spiked to lake water and imaged by WetSEM™. Figure 3.3 shows images of mixed metal nanoparticles spiked to lake water and imaged by conventional and WetSEM™. Figure 3.3a shows a dried sample revealing TiO_2 particles with sizes of 400 to 1000 nm; elemental

composition was confirmed by EDS. Particles are apparently surrounded by natural organic matter and other lake water components such as Na, Cl, Ca (confirmed by EDS). However, as the sample was in a dry state, artefacts cannot be omitted and information on the association of the TiO_2 particles with other lake water components as suggested by the EDS analysis could therefore be biased.

Figure 3.3b shows another area of the metal mix in lake water. Spiked Fe_2O_3 or ZnO particles could not be singled out compared to theTiO_2 particles in figure 3.3a, although EDS reveals the presence of Fe and Zn among *e.g.* Mg, Ca and Cl. However, Fe and Zn could also be part of the original lake water components and be picked up by EDS in the form of crystallised salts due to drying.

These possible drying artefacts have also been described by *e.g.* Doucet *et al.* (2005a), although using an ESEM to visualise natural aquatic particles and colloids. Doucet *et al.* (2005a) found that imaging under 100 % relative humidity was not possible in an ESEM as the hydrated samples were fully covered by a layer of free water. On the other hand, even partial drying of the sample by decreasing humidity was found to be able to induce shrinking and aggregation of the sample, which means that particle size can be significantly affected. Redwood *et al.* (2005) described similar effects of dehydration and salt crystal formation in conventional SEM and also ESEM images (even at high humidity) of humic substances. This limits the ability of these conventional methods for imaging unperturbed samples (Redwood *et al.* 2005). The presence of high concentrations of water has also been shown to hinder the imaging of humic substances (Redwood *et al.* 2005).

In comparison to the images obtained by conventional SEM, WetSEM^{TM} images of the spiked lake water samples clearly showed single TiO_2 particles (figure 3.3c) and also (quite diffuse) particle clusters (figure 3.3d) with a particle size of ~ 1 μm and therefore larger than in the ones visualised in the dried sample (figure 3.3a). However, attachment of the particles to the membrane was weak and therefore objects close enough to the membrane, thus suitable for imaging, were difficult to find. According to EDS analysis other lake water components were not detected in the immediate area around the particles.

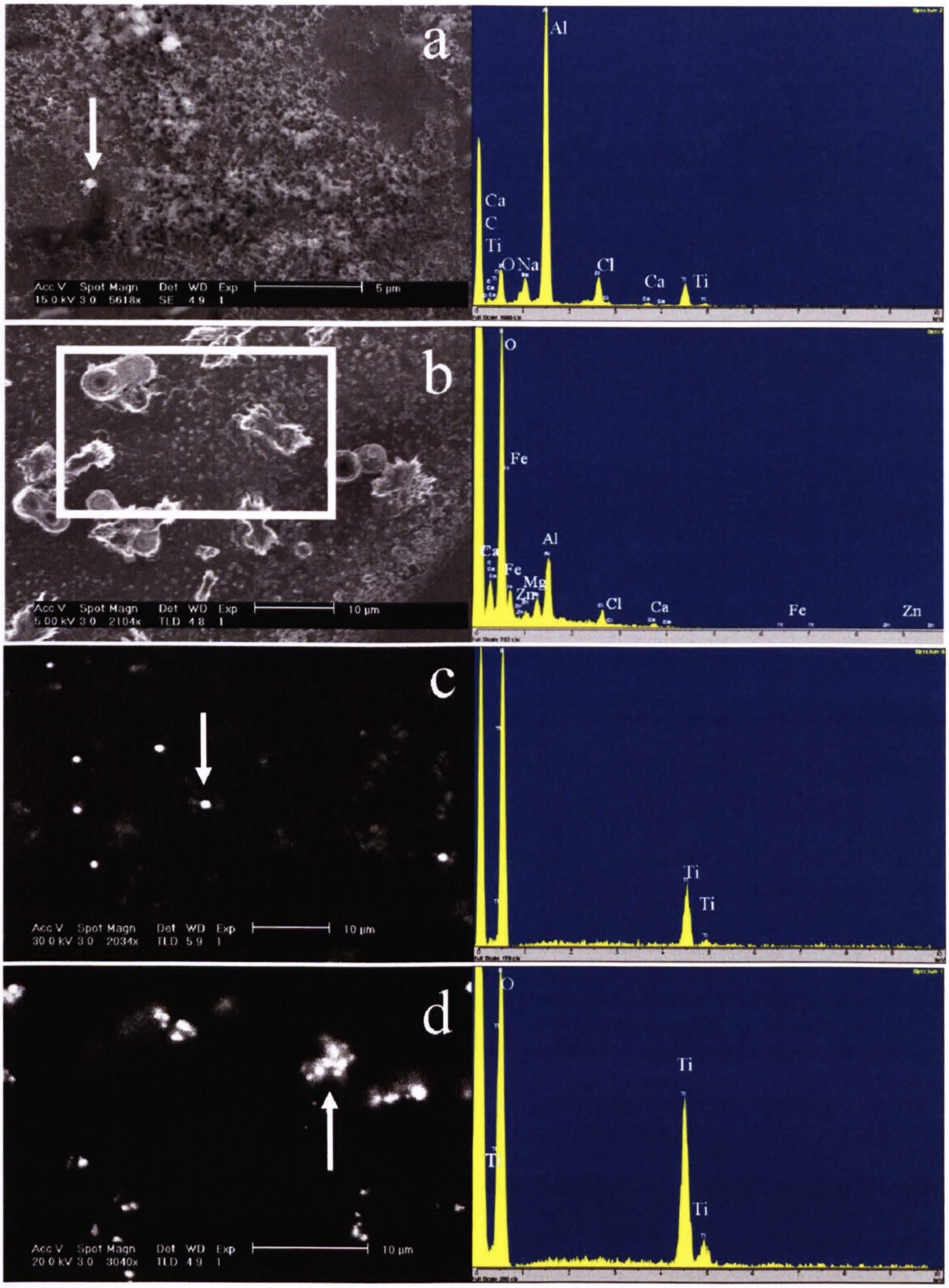


Figure 3.3. ZnO, Fe_2O_3, TiO_2 nanoparticles spiked to lake water and imaged by conventional SEM (a: scale bar 5 µm; b: scale bar 10 µm). WetSEM™ images of fully submerged, unperturbed metal oxide particles in lake water are shown in 3.3c, d (non treated capsule membrane; scale bar: 10 µm). EDS spectrograms are given for each image. Acquired X-ray spectra are indicated by the square box or arrows.

Capsule membrane coating for improved WetSEM™ imaging of metal-based nanoparticles in lake water

To still avoid drying artefacts in conventional SEM or in an ESEM, but to improve imaging and therefore information gain by WetSEM™, the capsule membranes were coated as ideally objects of interest have to be close to the capsule membrane to be visualised. Thus, to improve attachment of particles to the membrane, different membrane coatings were tested.

Figure 3.4 shows images of metal-oxide nanoparticle spiked lake water samples and their respective EDS spectrograms obtained by WetSEM™ using different membrane coatings. In figure 3.4a, EDS analysis of the visualised object (~ 3 µm) attached to the gelatine treated membrane reveals the presence of Zn and Fe in association with other lake water components such as Na, Cl, P, Ca.

Figure 3.4b shows single Fe_2O_3 particles attached to a poly-l-lysine treated capsule membrane (theoretically attracting negatively charged particles). With a size of ~ 150 to 300 nm these Fe_2O_3 particles are the smallest particles observed in this WetSEM™ study.

A definite improvement compared to the use of untreated capsules could be achieved for PSS coated membranes (theoretically attracting positively charged particles). Again a lake water sample spiked with ZnO, TiO_2 and Fe_2O_3 particles was studied (figure 3.4c). Although, ZnO particles could not be seen, presumably natural organic matter interacting with particles consisting of Ti and Fe (confirmed by EDS) could be imaged (figure 3.4c). This could indicate that not only particles may interact with organic matter, but also that different particle types might associate in aquatic systems. It is believed that the imaging of natural organic matter suspended in lake water has been achieved for the first time. The deeper penetration of the electron beam into the liquid sample enabled the 3D visualisation of the organic matter. Particles were determined to be in the size range of ~ 350 to 1300 nm. Other natural lake water components apart from natural organic matter could not be observed in figure 3.4b and 3.4c.

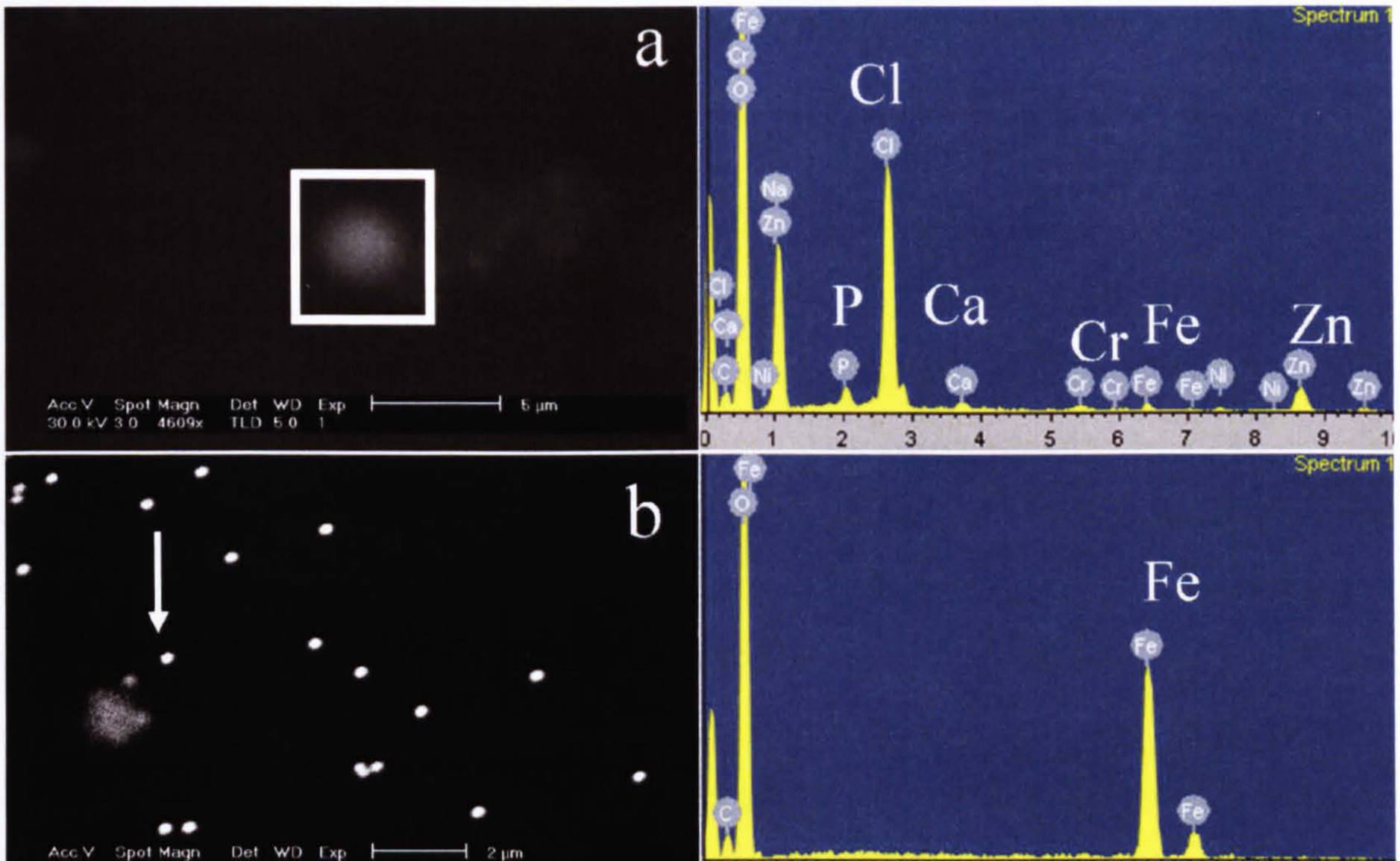


Figure 3.4. Images of a ZnO, Fe_2O_3, TiO_2 nanoparticles mixture in lake water using WetSEM™ capsules with coated membranes. Gelatine treated capsule membrane: scale bar 5 µm (3.4a); poly-l-lysine treated capsule membrane: scale bar: 2 µm (3.4b); PSS treated capsule membrane; scale bar: 10 µm (3.4c). Acquired X-ray spectra are indicated by the square box or arrows. EDS spectrograms show elemental composition of the respective samples. In 3.4c, TiO_2 and Fe_2O_3 particles visibly interact with natural organic matter as well as each other.

The capsules in combination with EDS only image and elementally detect particles, and therefore, in water, dissolved lake water components such as NaCl do not show in WetSEM™/EDS analysis in comparison to the salt crystals observed and analysed by conventional SEM/EDS. This could provide essential additional information to conventional SEM imaging.

Figure 3.4c shows that carbon or carbon-containing material such as natural organic matter can be visualised by WetSEM™, however, elementally identification by EDS

is not possible as the membrane of the capsules consists of a polymer and therefore C and O peaks are always observed.

WetSEM™ imaging of metal-based nanoparticles in a soil suspension

For imaging of Au particles in a soil suspension QX-302 capsules were used. For these, mechanical attachment of the sample to the capsule membrane was achieved with the help of spacers (number of spacers depending on the thickness of the sample). In figure 3.5 images of the spiked soil suspension are shown and particles in a size range of 300 nm to 20 µm can be observed. From conventional EM imaging of the applied Au particles in distilled or natural water, it was found that they do not tend to aggregate and keep their initial sizes of ~ 50 nm and also their spherical shapes. For these images EDS was unavailable and as the observed particles in figure 3.5 were bigger than 50 nm, it is assumed that the high atomic number Au particles interact with the soil particles (adsorb, coat), which then appear bright (figure 3.5).

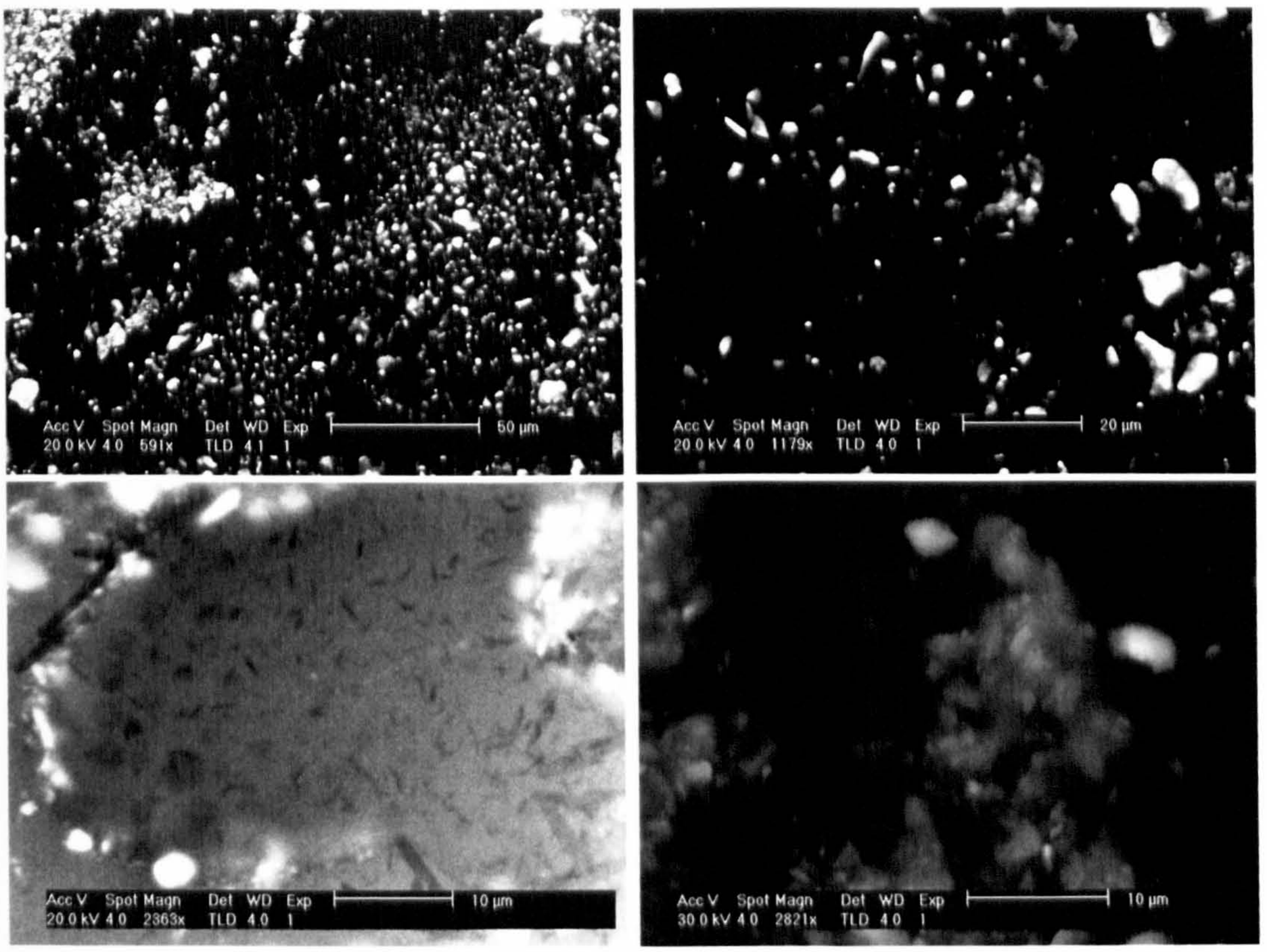


Figure 3.5. WetSEM™ images (different magnifications, scale bars indicate 50, 20, 10, and 10 µm) of a soil suspension spiked with Au particles (50 nm). Bright large areas indicate interaction of Au particles and soil particles.

Conclusions

To conclude, compared to conventional SEM, which show dehydration artefacts, WetSEM™ has potential for in-situ imaging of unperturbed environmental samples while avoiding many of the artefacts associated with other imaging techniques. As with other submicron microscopic techniques, the capsules allow qualitative observations on the major class of particles present in the sample; they can provide information on particle size and composition (in combination with EDS), state of aggregation and particle associations. In general imaging of particles with a higher atomic number (such as gold) facilitates imaging tremendously. Although the capsules are not reusable, their quick and easy application with minimal or no sample preparation in a conventional SEM is an advantage. Additionally, as the sample is in a closed capsule, imaging under standard pressure is possible (*e.g.* compared to ESEM).

However, there are a number of limitations of WetSEM™ that mean that it is probably not yet suitable as a routine method for environmental analysis. For example:

1) imaging through a membrane significantly reduces the spatial resolution of SEM, the membrane is also very sensitive to radiation damage;
2) as particles are able to move in the liquid through Brownian motion, images can be blurred;
3) the current detection limit of the method is lower than the imaging methods that involve a drying step (which concentrates the study particles) and is probably greater than concentrations expected in the natural environment;
4) imaging can be difficult, if particles do not attach to the capsule membrane as electron scattering and therefore object detection worsens with the distance of the object of interest from the membrane. In addition, as only the top surface of the sample is imaged, the representativeness of the whole sample is unknown;

5) the approach can currently only provide qualitative information and as the method is relatively costly and time-consuming, it is not possible to visualise large numbers of samples or characterise large numbers of particles within a sample.
6) in the current study, the method has only been shown to be appropriate for metal and metal oxide nanoparticles and its application to a wider range of particle types is unknown.

Many of these limitations can likely be addressed through further developmental work. For example, object attachment to the membrane can be improved by membrane coating; pre-concentration methods may increase the sensitivity of the approach (although these could also introduce artefacts); and via evaluation studies employing a number of different characterisation techniques on different samples.

It is believed that the ability of visualising untreated, wet nanoparticle samples is a significant achievement and thus WetSEM™ can supply significant supplementary information on the in-situ investigation of particles in environmental matrices. Therefore, in combination with complementary techniques (SEM, TEM, AFM) and other analytical tools (*e.g.* HDC- or FFF-ICP-MS, DLS), WetSEM™ could help to provide a better understanding of the environmental fate and behaviour of ENPs in the future.

However, while the method produces useful information, it is expensive and highly time consuming and probably needs significant development before it can be used routinely in environmental studies. Therefore it has not been used in the fate investigations described later in this thesis.

Chapter 4

Size characterisation of inorganic nanoparticles in 'real' environmental samples using HDC-ICP-MS

Introduction

This chapter describes the validation of a novel chromatographic technique, hydrodynamic chromatography (HDC), which not only separates particles over the conventional size range of nanoparticles, *i.e.*, from 5 to > 100 nm, but goes well above this (the column used in this work goes up to 300 nm, but other formats can go to ~ 500 nm). This extended sizing range should allow researchers to monitor processes such as particle aggregation, particle/natural organic matter interactions, and also particle dissolution. When interfaced to a multi-element detector (ICP-MS), the technique allows investigations into the behaviour and fate of a range of inorganic ENPs in 'real-world' situations.

In HDC, particle separation is solely based on particle size, and is independent of particle type and density (Yegin and Lamprecht 2006). The column is packed with non-porous beads, which build up flow channels or capillaries. Particles are separated by flow velocity and the velocity gradient across the particle (Mcgowan and Langhorst 1982; figure 4.1). In the narrow conduits, larger particles are transported faster than smaller ones, as they cannot fully access slow-flow regions near the conduit walls (Small *et al.* 1976), leading to faster elution of larger particles from the column, and higher retention times for smaller particles.

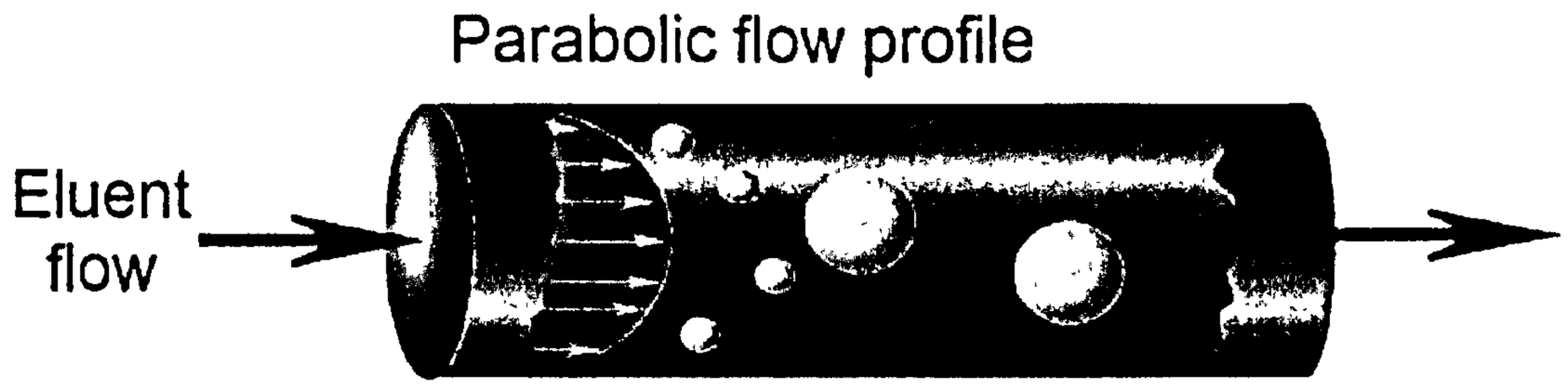


Figure 4.1. Primary separation mechanism in the HDC column; separation by flow velocity in channels between particles

A number of approaches have been proposed for the detection and characterisation of ENPs in aquatic samples, including microscopy-based techniques (chapter 3), light scattering methods and several based on chromatography (chapter 2). The most promising of these involve the use of separation techniques such as field flow fractionation (FFF) (Baalousha *et al.* 2005a; Baalousha *et al.* 2005b; Chen and Beckett 2001; von der Kammer *et al.* 2005b; Baalousha *et al.* 2006a; Baalousha *et al.* 2006b; Siepmann *et al.* 2004; von der Kammer *et al.* 2004), liquid chromatography (Arangoa *et al.* 2000; Song *et al.* 2004; Wilcoxon and Provencio 2005; Wilcoxon *et al.* 2001; Wilcoxon *et al.* 2000; Sivamohan *et al.* 1999; Song *et al.* 2004; Song *et al.* 2003; Saridara and Mitra 2005), size exclusion chromatography (SEC) (Bolea *et al.* 2006; Bootz *et al.* 2005; Huve *et al.* 1994; Krueger *et al.* 2005; Liu and Wei 2004; Wei and Liu 1999; Wang *et al.* 2006), gel electrophoresis (GE) (Bruchert and Bettmer 2005), and capillary electrophoresis (CE) (Schnabel *et al.* 1997; Schmitt-Kopplin and Junkers 2003; Lin *et al.* 2007; Feick and Velegol 2000). Where these have been combined with element-specific detectors, such as ICP-MS (Hassellöv *et al.* 1999b; Giusti *et al.* 2005; Helfrich *et al.* 2006; Metreveli *et al.* 2005; Siepmann *et al.* 2004), an additional degree of selectivity is gained, thereby increasing the quality of the data obtained. To our knowledge, only FFF-ICP-MS has been successfully applied to samples in environmental media (*e.g.* Gimbert *et al.* 2007a; Stolpe *et al.* 2005), the others having only been used on standards and/or simple solutions. The limitations of the different separation techniques are *e.g.* the complexity and time-consumption as well as membrane interactions and membrane cut-off of FFF (appendix 2), the limited size separation range of available and solid phase

interactions of SEC columns (Lead and Wilkinson 2006), and the complex interpretation of migration times, *i.e.*, distinguishing size-based from non-size-based interactions (CE and GE).

In addition to the advantageous particle sizing range available when using HDC, the method developed in this chapter also utilizes the full capability of state-of-the-art ICP-MS collision-cell technology (simultaneous analysis of six particle types, based on TiO_2, SiO_2, Au, Ag, Al_2O_3 and Fe_2O_3). The stabilisation (in a range of environmentally-relevant matrices) and use of Au particles, both as external calibrants (5 to 250 nm) and as internal standards (the latter, improving the accuracy of particle sizing data by allowing relative retention times (rRT) to be calculated) are also presented. The analytical method was evaluated for a range of ENPs in different sample matrices. The method features a high sample throughput, with minimal sample preparation and it is believed that this approach is suitable for the routine size and elemental characterisation of inorganic ENPs in environmental fate and ecotoxicity studies.

Materials and methods

Analytical Method

Size separation was achieved using a PL-PSDA Type-1 hydrodynamic chromatography column (separation range 5-300 nm) (Polymer Labs, Shropshire, UK) attached to an Agilent 1200 HPLC system (Agilent, Cheshire, UK) fitted with an auto-sampler (figure 4.2).

The water-based mobile phase was a proprietary product comprised of a salt mixture and surfactants, and was obtained from Polymer Labs (Shropshire, UK). As a proprietary product, exact details of its composition are not available. However, a comparable HDC mobile phase was described by Mcgowan & Langhorst (1982) and comprised 0.002 M Na_2HPO_4, 0.2 % non-ionic surfactant, 0.05 % $C_{12}H_{25}SO_4Na$ (SDS), 0.2 % formaldehyde, adjusted to pH ~ 7.5. The flow was split, post-column, using a tee joint to decrease the sample volume reaching the ICP-MS plasma and to

allow natural aspiration of the nebulizer. The injection volume was 20 µL (unless otherwise stated); the mobile phase flow rate was 1.7 ml/min; pressure was constant at ~ 9 MPa. All tubing was made of inert material. Detection was achieved using an Agilent 7500 ICP-MS (Agilent, Cheshire, UK). The instrument set-up and parameters are given in table 4.1.

The Agilent ICP-MS allows the analysis of elements normally affected by plasma-based polyatomic interferences, by adding different gases, *e.g.*, helium and hydrogen, to a collision cell. Therefore, using the instrument in hydrogen mode, it was possible to simultaneously analyse problematic particles, such as Fe_2O_3 and SiO_2 alongside TiO_2, Al_2O_3, Ag and Au nanoparticles. To ensure that the separated peaks were due to the element of interest, and not residual interferences, additional isotopes (where available) were also monitored. The ratio of the element's isotopes could then be used to confirm the elemental identity of the peak.

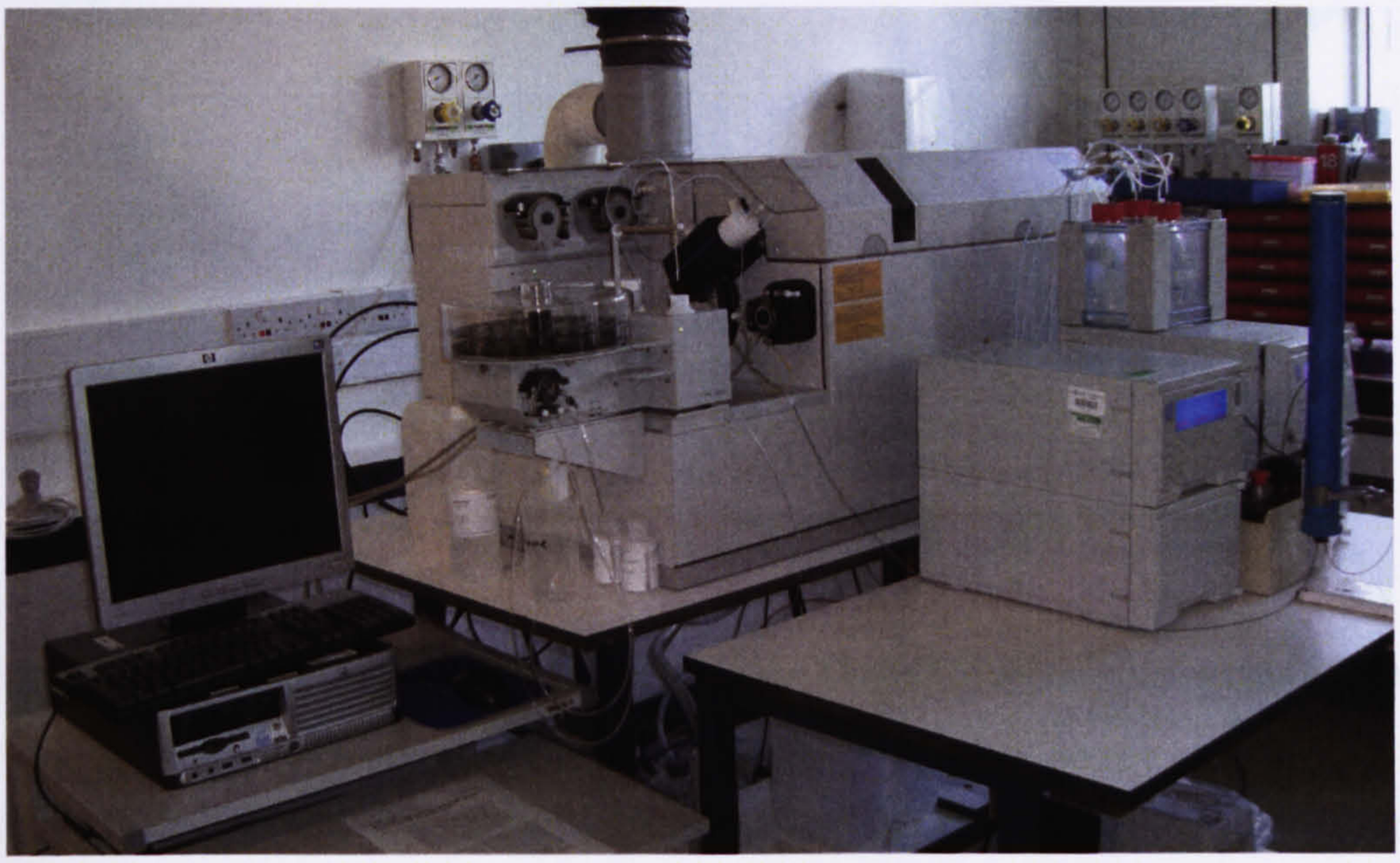


Figure 4.2. Picture of HDC-ICP-MS set-up.

Table 4.1. Instrumental parameters for the ICP-MS.

Parameter	*Setting*
RF power	1550W
Reaction cell mode	Off On: H_2 gas: 4.9 mL/min (Ti, Fe)
Nebulizer pump	0.3 rps
Nebulizer gas flow:	
Carrier gas	0.75 L/min
Make-up gas	0.34 L/min
Sample depth	8 mm
S/C temperature	2 degC
Extract 1	0 V
Extract 2	-100 V
Omega Bias-ce	-18 V
Omega Lens-ce	0.8 V
Cell entrance	-36 V
QP focus	5 V
Cell exit	-36 V
OctP RF	190 V
OctP Bias	-7 V
QP Bias	-4 V
Isotopes monitored	^{27}Al, ^{28}Si, ^{47}Ti, ^{48}Ti, ^{56}Fe, ^{107}Ag, ^{109}Ag, ^{197}Au

Size calibration

Selection and validation of size calibration standards. Gold particles were chosen as ICP-MS calibrants because the element is rare in nature, mono-isotopic (giving maximum sensitivity from a small added amount), and is relatively interference-free. In addition to this, the particles are readily commercially-available as well-characterised spheres, covering an appropriate size range, with relatively narrow size distributions. Gold particles, of nominal mean diameters; 5 nm (range 3.5-6.5 nm); 10 nm (range 8-12 nm); 20 nm (range 17-23 nm); 50 nm; 100 nm and 250 nm, were used. The smaller particles (5-20 nm) were obtained from Sigma Aldrich (Dorset, UK) pre-dispersed in 0.01 % tannic acid with 0.04 % trisodium citrate, 0.26 mM

potassium carbonate and 0.02 % sodium azide (the latter as a preservative). The 5 and 10 nm gold particles were filtered through 20 nm Anatop filters (Whatman International Ltd, UK) to improve particle homogeneity. The larger particles were obtained from BBInternational (Cardiff, UK).

The average diameters of the differently sized gold nanoparticle standards were confirmed by automated object-based analysis of transmission electron microscopy (TEM) images. For TEM analysis, samples were prepared by applying droplets of the respective samples to 200 mesh copper grids with carbon film (Agar scientific, UK) and allowed to dry. Imaging took place on a JEOL 2010 TEM coupled to energy dispersive x-ray spectrometry (Oxford Instruments, UK), using standard conditions (200 kV acceleration voltage). Image analysis of at least ten representative images from five different grids was carried out using Definiens Developer^{TM}, an automated object-based image analysis software (Tiede *et al*. 2008; Athelogou *et al*. 2007). The Definiens Developer software allows automated classification of image objects (created through segmentation) and the calculation of statistical values such as mean size, length and width relations of the extracted objects. Rule sets for image object classification are developed in a modular programming language (CNL – Cognition Network Language), which enables the transferability to other images and leads to a significant performance gain compared to manual image analysis (Tiede and Hoffmann 2006).

Steric stabilisation of gold nanoparticles for internal retention time marking in environmental matrices. To prevent potential destabilisation and aggregation of the electrostatically-stabilised gold particles when spiked as internal standards in high ionic strength samples, *e.g.* natural water samples, the calibrants were sterically stabilised. Note, if the particles are only to be used as external calibrants, stabilisation is not required. Steric stabilisation was performed by first linking an amine reactive derivative of biotin to a 70,000 MW dextran polymer and then conjugating the polymer to the gold particle. This procedure was chosen not only because of the increased stability of the resulting particles, but also because the researchers reported that the added layer had negligible effect on the particle's

diameter (Chen *et al*. 2004; Wilson *et al*. 2004). Their explanation was that the linear relationship between the minimum numbers of molecules required to prevent flocculation and the square of the particle diameter, suggested that the surface of the particle had become totally enveloped by the dextran.

Evaluation of the stability of sizing standards in different media. To explore the stability of the gold particle standards in a range of media that could be used in environmental fate and behaviour studies, the stabilised and non-stabilised gold particles were added (to give a final concentration of ~ 5 mg/L) to distilled water (Millipore, UK); lake water sampled from a lake on the grounds of the Central Science Laboratory (CSL, Sand Hutton, UK); a 0.01 M $CaCl_2$ solution ($CaCl_2$ matrix is recommended for use in environmental fate studies by the OECD (2000)); and the HDC-ICP-MS mobile phase, and then left for at least 24 hours. Samples were prepared for TEM imaging as described above. TEM/EDX was applied, under standard operating conditions, to validate the sizes and stability of the different gold particles in different media.

Reproducibility and robustness of the HDC-ICP-MS methodology. To ensure the reproducibility and robustness of the HDC-ICP-MS approach, the gold calibrants were analysed, as bracketing standards, between every fourth sample injection within an analytical batch (*i.e.*, n = 4 for each particle type, per analytical batch). Between-run reproducibility for each gold particle was assessed from the above analyses, performed over a 6-week period (n = 32).

Size calibration approach. Size calibration of the HDC column was performed using the six gold standards. Mean diameters of the particles were plotted against retention times, to obtain a calibration curve. Calibration curves were constructed at the start, middle and end of each analytical run, with samples bracketed by injections of a single standard (20 or 50 nm, depending on the sample). Because of the lateral flow processes involved in the separation mechanism, the resulting calibration curves for HDC are not linear. Whichever single standard was used as the bracket calibrant, it

was also used as an internal standard where possible, to monitor matrix-induced retention time shift.

Evaluation of analytical method for a range of ENPs in different sample matrices

In order to assess the suitability of the method for use with 'real' environmental samples, silver particles (nominal size < 100 nm, 10 % wt, Sigma Aldrich, Dorset, UK) were spiked into sewage sludge (Broadholme Sewage Treatment Works, near Wellingborough, Northamptonshire, UK) to give concentrations of approximately 10 mg/L in 2 g/L of mixed liquor suspended solids (MLSS). The mixture was then shaken for 6 hours, allowed to settle and the supernatant then directly analysed by HDC-ICP-MS (injection volume 100 µL), without the need for filtration, centrifugation, or any other preparative step. The samples were also analysed by TEM, to validate the results obtained from the HDC-ICP-MS.

To assess the suitability of the method for the simultaneous analysis of different nanoparticle types, a mixture of metal and metal oxide ENPs was analysed, using external calibration, and a 20 nm gold internal standard (retention time marker). The following nanoparticle powders and dispersions were purchased from Sigma Aldrich. Particle sizes were given by the manufacturer as follows: Fe_2O_3 powder (20-25 nm); Al_2O_3 dispersion in water (< 20 nm); TiO_2 powder (5-10 nm); SiO_2 alumina doped (< 20 nm). Nanoparticle powders were suspended in Milli-Q water (Millipore, UK) at concentrations of ~ 5 mg/L and shaken over night. The internal standard was added to the mixture and the resulting spiked Millipore water was then analysed by HDC-ICP-MS. Images of the metal mixture in distilled water were additionally taken by TEM (same instrument, instrument conditions and sample preparation as described above; image analysis was performed by ImageJ).

Results and discussion

Size calibration

Selection and validation of size calibration standards. In the first stage of this work, a number of gold particles were selected for use as size calibrants for the HDC column. The average particle size and size distribution of these standards was validated by TEM and automated object-based image analysis. The TEM data confirmed that the mean diameters were in good agreement with the manufacturer's sizing data (table 4.2). With the exception of the 250 nm standard, the gold particles showed a relatively low degree of polydispersity, with standard deviations being < 1 of the mean size. However, even though this value is deemed good (in terms of the particle manufacturing process) it would benefit from being even smaller, when being used in the context of size calibration standards.

Table 4.2. The average diameters of the gold particle standards in nm, as stated by the manufacturers, with the average diameters and standard deviation (σ) as measured by TEM and image analysis.

Manufacturer's data				*TEM & image analysis*	
Nominal diameter	Mean diameter	Coefficient of variation	Number conc. (per mL)	Mean diameter	Standard deviation
5	3.5-6.5	< 15 %	4.0×10^{13}	5.2	0.55
10	8.0-12.0	< 15 %	5.0×10^{12}	9.4	0.64
20	17.0-23.0	< 15 %	5.5×10^{12}	24.8	0.29
50	49.7	< 8 %	4.5×10^{10}	44.6	0.98
100	101.6	< 8 %	5.6×10^{9}	111.1	0.58
250	261.2	< 8 %	3.6×10^{8}	261.1	45.1

Evaluation of the stability of sizing standards in different media. As the calibrants were intended for use with samples arising from environmental ecotoxicology studies, their stability in solutions representative of those encountered in such studies

was evaluated over a 24 hour period, *i.e.*, distilled water (Millipore, UK); lake water sampled from a lake on the grounds of the Central Science Laboratory (CSL, Sand Hutton, UK); a 0.01 M $CaCl_2$ solution; and the HDC-ICP-MS mobile phase.

According to the analysis of the TEM images, all the particles were stable in the various test solutions (single particles clearly distinguishable) except the non-stabilised particles in the lake water and $CaCl_2$ matrix (particles show aggregation: single particles not always distinguishable, however, imaging artefacts such as overlaying cannot be excluded). The destabilisation is thought to be due to the relatively higher ionic strength in the $CaCl_2$ and lake water matrices (there are no divalent cations in the mobile phase) affecting the electric double layer of the particles, resulting in a decrease in the stabilising electrostatic repulsive forces (Fischer and Kenndler 1997; Wei and Liu 1999). Because the $CaCl_2$ matrix is routinely recommended for use in environmental fate studies (OECD 2000), researchers wishing to use these particles as internal standards should stabilise them accordingly before use. Figure 4.3 shows typical TEM images for two of the particle types (non-stabilised 50 nm and stabilised 50 nm particles), in the various media, and shows the effectiveness of the stabilisation process in the matrices assayed.

Size calibration approach. In general, peaks for the different sized gold nanoparticle standards were reproducible and symmetrical (see figure 4.4). The exceptions were the 100 nm standards, which gave an asymmetric, but reproducible peak, and the 250 nm particles, which produced a relatively broad peak (figure 4.4). These results probably reflect their greater polydispersity compared to the smaller particles. No differences in retention time were observed for stabilised and non-stabilised particles (tested on stabilised and non-stabilised 50 nm Au particles).

Figure 4.4 and 4.5 present typical HDC size calibration curves, (with error bars indicating standard deviation associated with each point). However, as can be seen, these error bars are not easily discernable, as the standard deviation associated with the relative retention time for each particle is very small, *i.e.,* 5 and 50 nm = 0.12, 10, 20 and 100 nm = 0, and 250 nm = 0.33 (n = 4). From this, it can be seen that,

although the absolute retention time window is not large, the quality of the particle sizing data obtained is achievable because of the precision of the elution behaviour. The quality of the particle sizing data is totally dependent on the accuracy and precision of the measured retention time. Therefore, gold calibrant particles were additionally added to samples, for use as internal standards (retention time markers). This was an effective approach to ensuring the quality of the sizing data, because it allowed relative retention times (rRT) to be calculated.

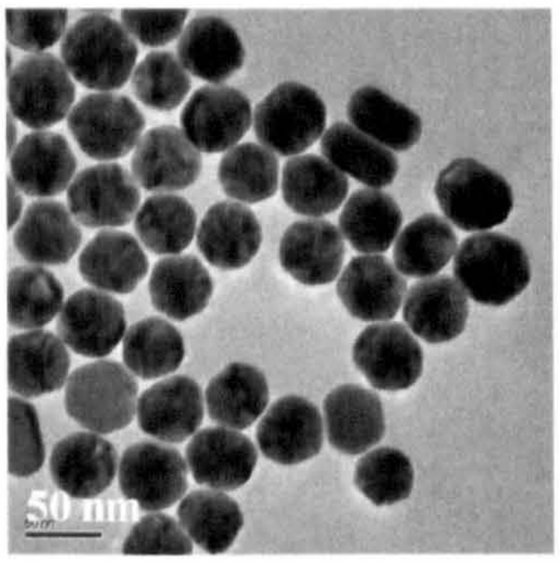
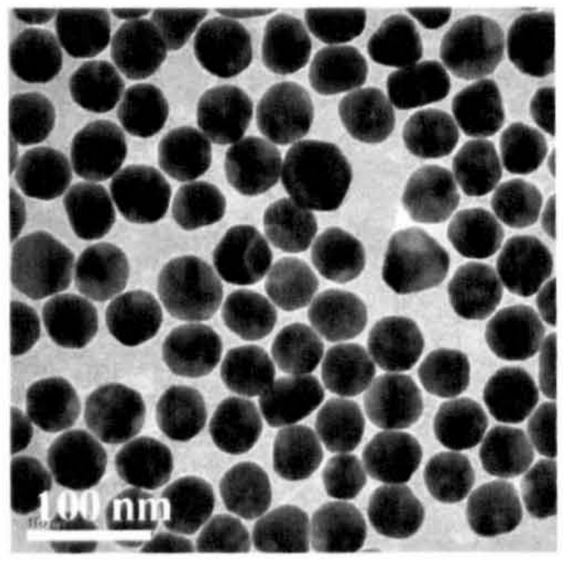
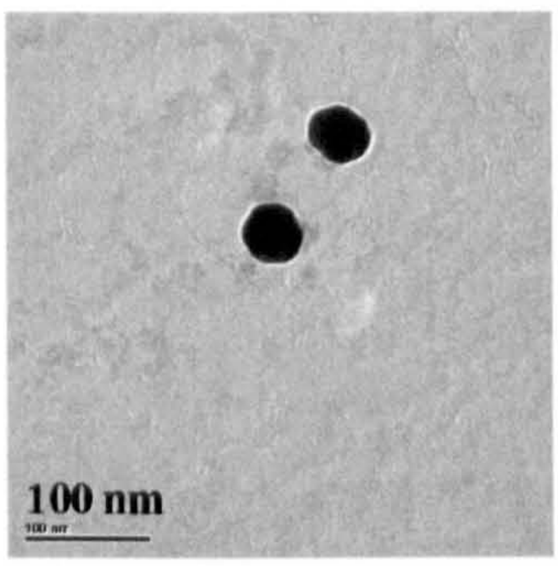
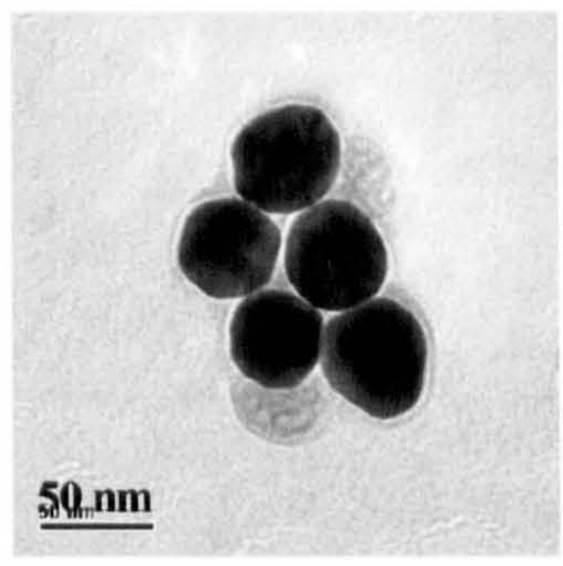
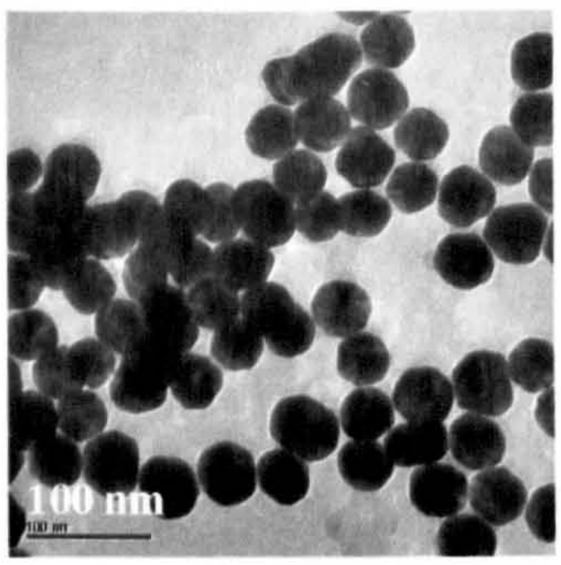
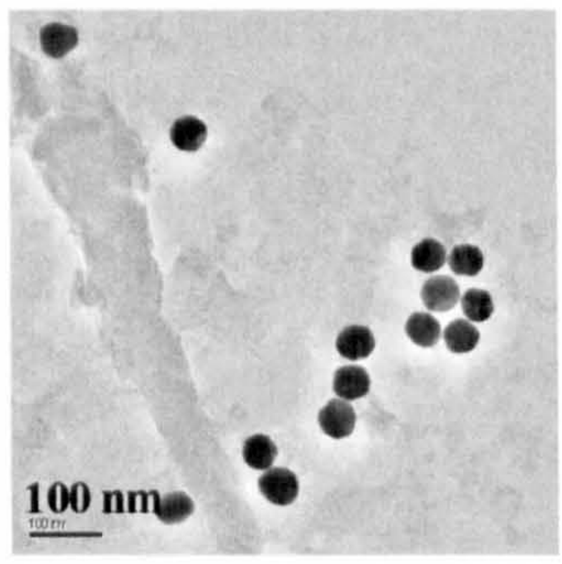
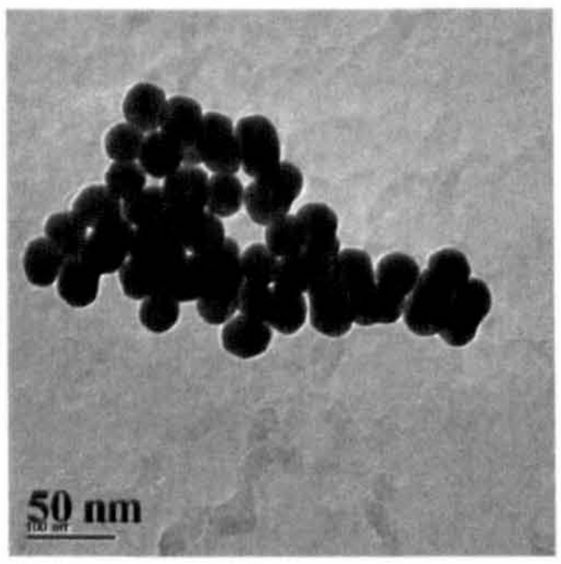
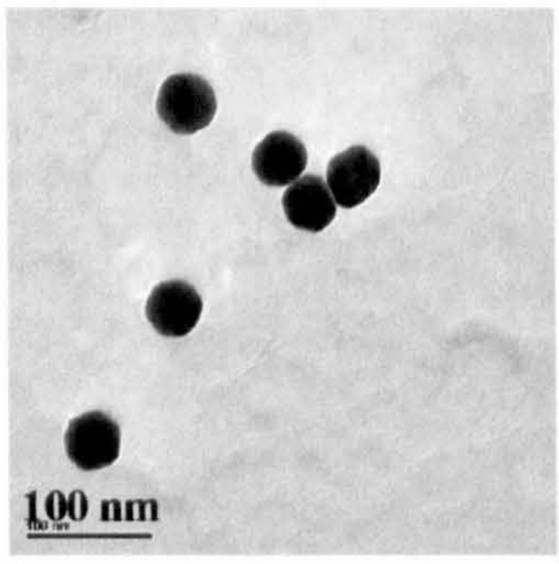
	Au 50 nm (non-stabilised)	Au 50 nm (stabilised)
Distilled water		
Mobile phase		
Lake water		
$CaCl_2$		

Figure 4.3. Representative TEM images of colloidal gold 50 nm (non-stabilised) and 50 nm (stabilised) in distilled water, HDC-ICP-MS mobile phase, lake water and $CaCl_2$.

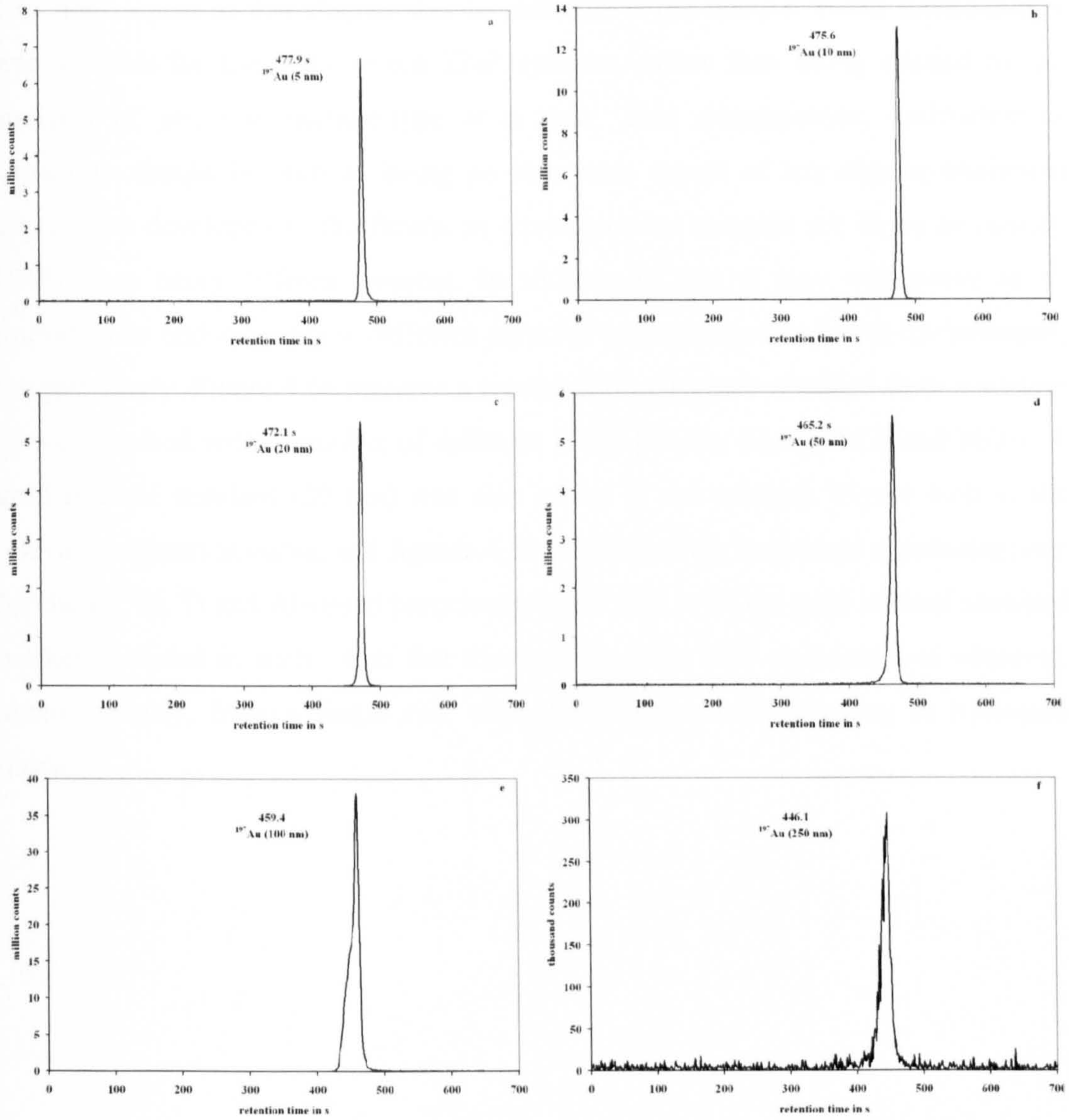
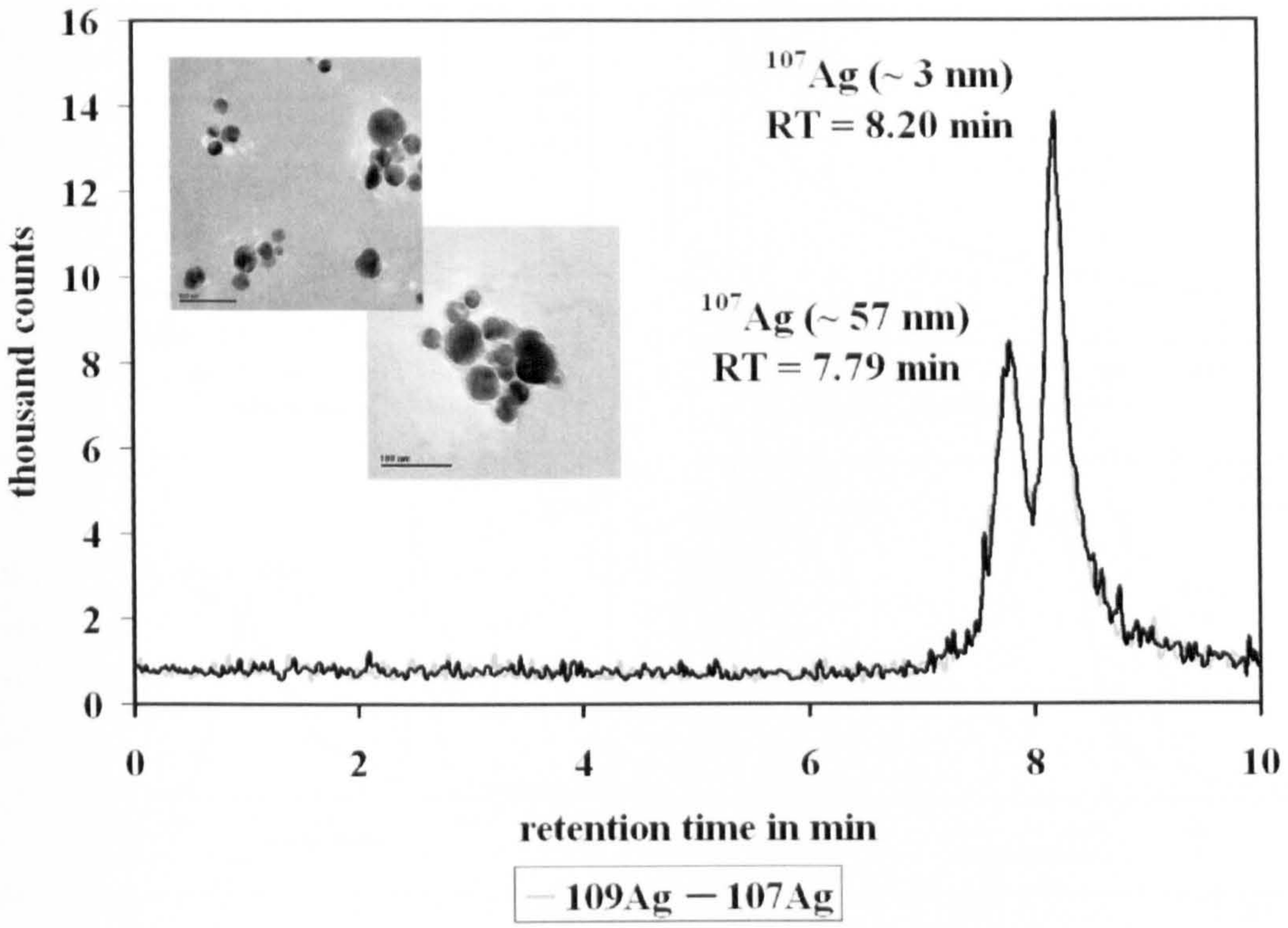


Figure 4.4. Typical ICP-MS chromatograms of (a) 5 nm, (b) 10 nm, (c) 20 nm, (d) 50 nm, (e) 100 nm and (f) 250 nm nominal sized gold particles.

Evaluation of analytical method for a range of ENPs in different sample matrices

Having established the quality of the separation procedure, the hyphenated system was applied to a 'real' environmental matrix, *i.e.*, sewage sludge supernatant. Figure 4.5 presents a typical chromatogram and TEM images obtained from an investigation into the behaviour of silver nanoparticles in sewage sludge, which will be discussed in chapter 5.

The final aspect of this chapter was to establish if the HDC-ICP-MS methodology was suitable for use with mixed ENP systems, rather than being limited to the analysis of just one particle-type at a time. This simultaneous, multi-element capability should be seen as being an important aspect of any similar analytical approaches developed in the future, as environmental samples are likely to contain ENPs from many different sources. In addition to this, it may well prove to be important to understand how different particles interact together in the environment, not just singly. Figure 4.6a presents a typical chromatogram obtained from a sample of water spiked with a number of different ENPs (Fe_2O_3, Al_2O_3, TiO_2 and SiO_2). A gold internal standard (20 nm) was also added to the mixture. Figure 4.6b is the relevant calibration curve, and figures 4.6c to 4.6e are the individual chromatograms for the Fe, Si, Ti and Al-based particles, respectively, with the gold internal standard marker included in each. Note that the data for these five elements was obtained, simultaneously, from a single run, with the collision cell operating in hydrogen mode.



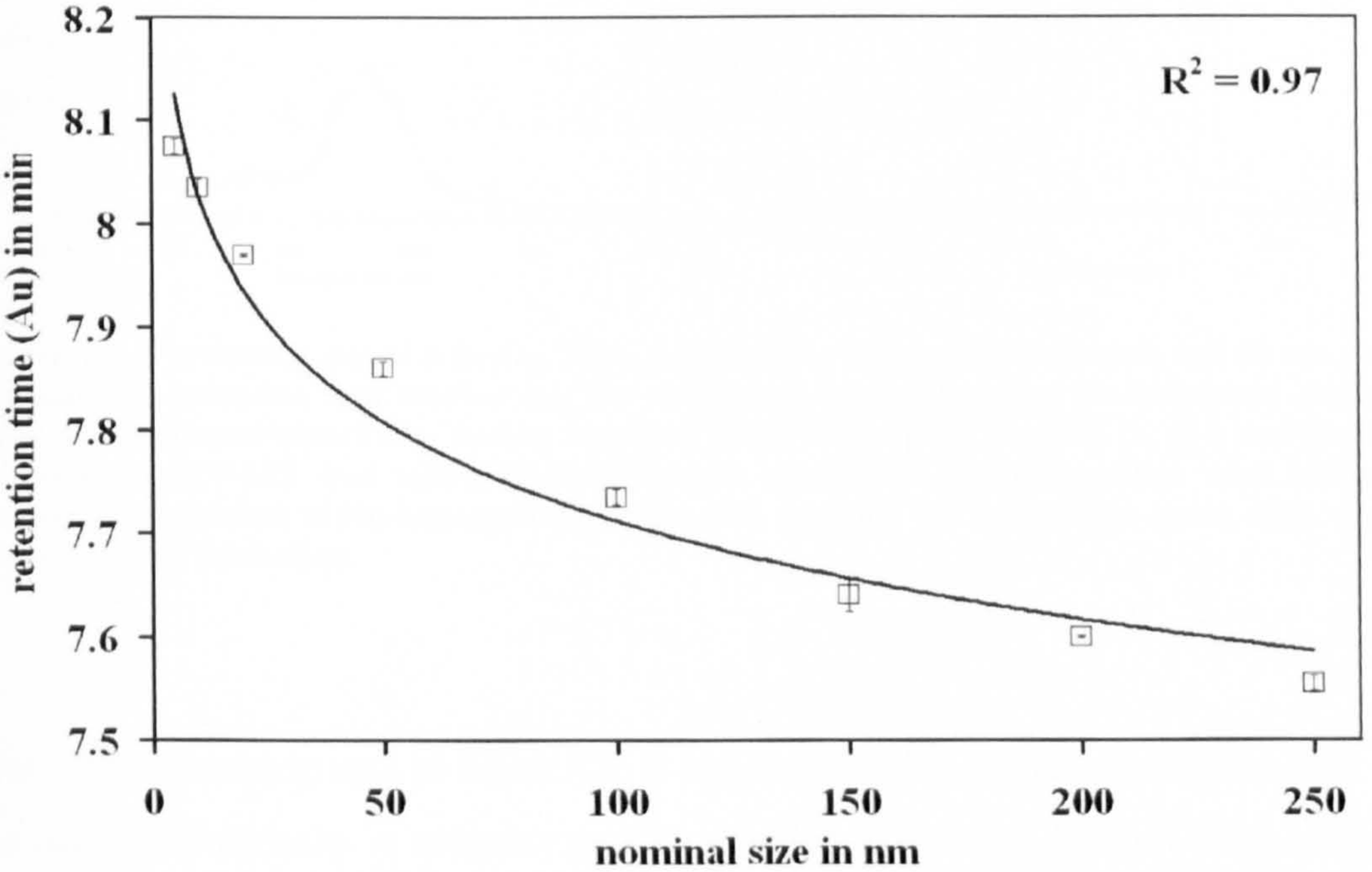


Figure 4.5. HDC-ICP-MS chromatogram and TEM images obtained from a supernatant sample of sewage sludge spiked with silver nanoparticles (10 mg-Ag/L, 2 g/L MLSS) and corresponding calibration curve (error bars indicate standard deviation of retention times; here gold calibration standards with averages sizes of 150 and 200 nm, BBI UK, were additionally used).

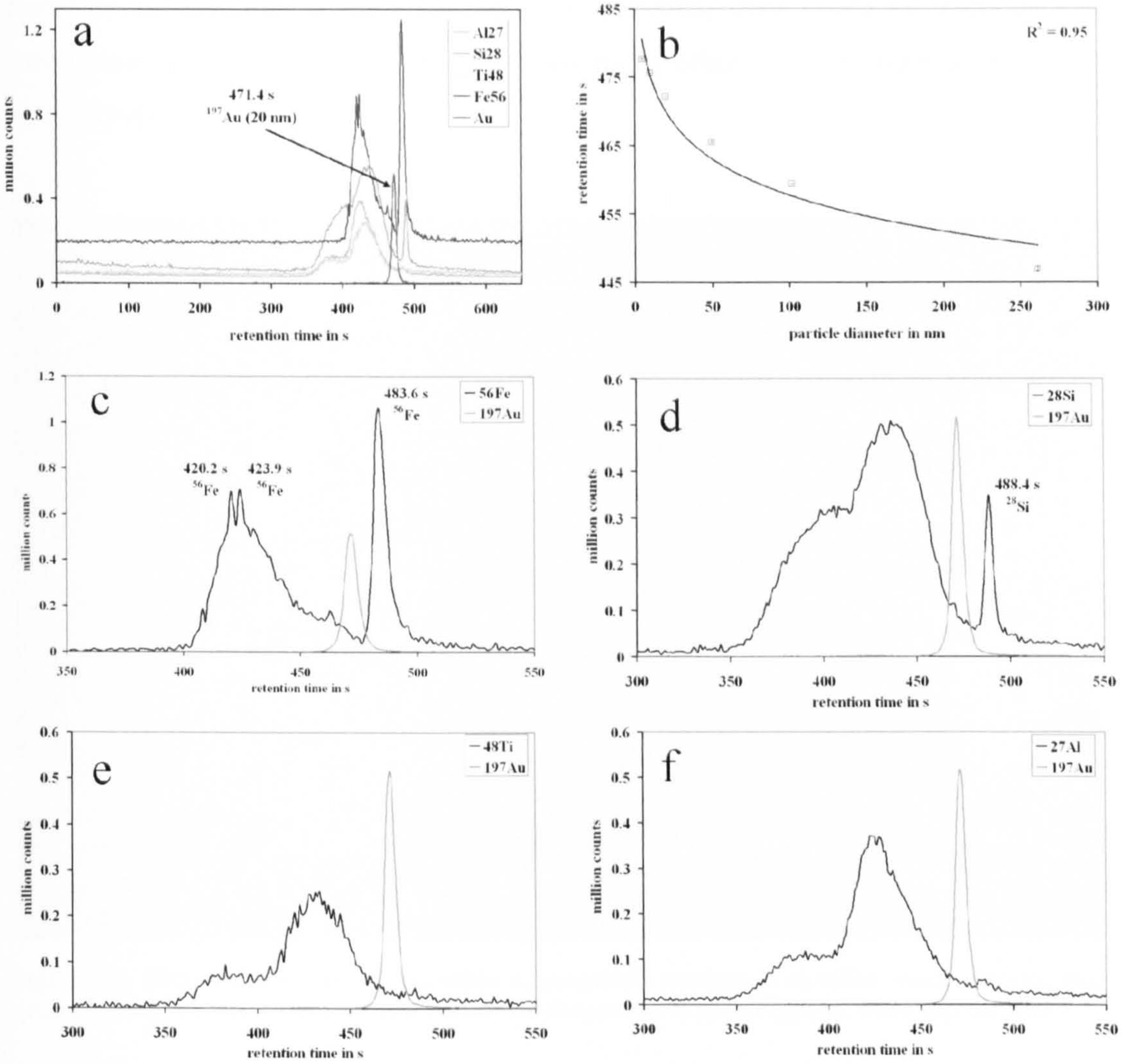


Figure 4.6. Chromatogram of a Fe_2O_3, TiO_2, Al_2O_3, SiO_2, nanoparticle mixture and 20 nm gold I/S, added as retention time marker (a). For clarity, the chromatograms for individual particle types are presented separately, stating retention times of the peak maxima (c, d, e and f). The collision-cell ICP-MS was operated in hydrogen gas mode. Retention times and isotopes monitored are stated in chromatogram. Figure 4.6b presents the calibration curve with error bars (standard deviation).

From the chromatograms in figure 4.6, it can be seen that the retention behaviour of the individual particles is complex making detailed discussion difficult. This is most likely due to their high polydispersity observed by TEM (figure 4.7).

However, it can be stated from the chromatograms that there is no interaction between the gold internal standard and any of the particles or other components in the water, providing strong support for their suitability as internal standards. It has been shown that the gold nanoparticles are stable under a range of conditions,

therefore in addition to their use in HDC-ICP-MS, they could be used as size calibration and internal retention time markers in other methods such as FFF- and SEC-ICP-MS.

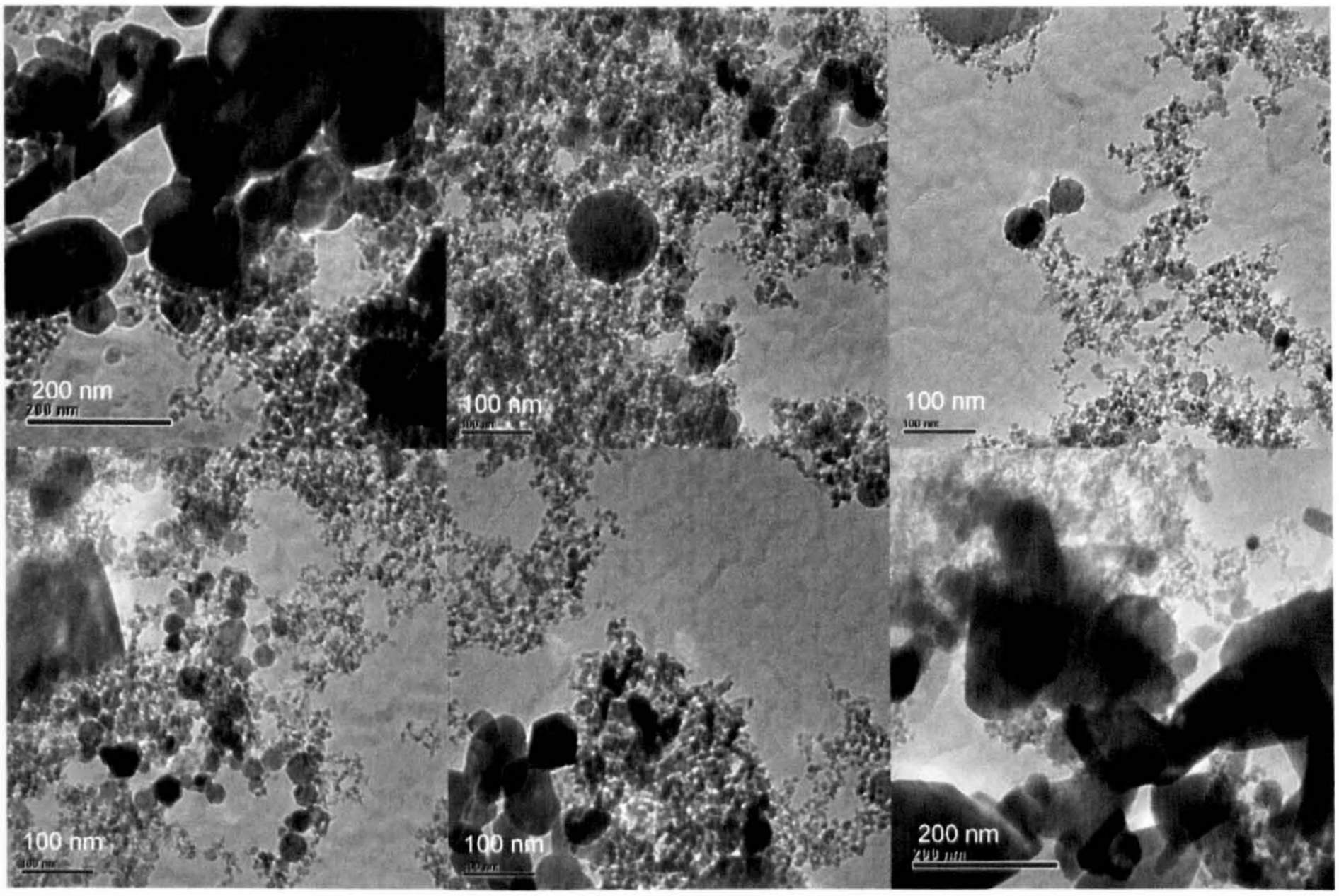


Figure 4.7. TEM images of the metal oxide nanoaprticle mixture in distilled water showing high polydispersity (~ 3 to ~ 250 nm) and variety of shapes. EDX spectrograms were unavailable.

The iron and silica-based oxide particle systems display discreet peaks (483.6 s (~ 8.06 min) and 488.5 s (~ 8.14 min), respectively) for the added material, these peaks are below the 5 nm calibration mark and therefore outside the calibration range of the gold standards (particles below 5 nm can also be observed in the TEM images in figure 4.7). Only a small fraction of the overall signal response for the study nanoparticles was within the calibration range (5 - 250 nm). All systems (TiO_2, SiO_2, Al_2O_3, and Fe_2O_3) showed that the greatest signal response above the maximum calibration size (250 nm). This is supported by TEM analysis, which showed particles or aggregates of 250 nm and above (Figure 4.7). These data are in agreement with other studies showing aggregation of *e.g.* TiO_2 and SiO_2 particles in water where size ranges of 175-810 nm (mean 330 nm) were reported for TiO_2 and 135-510 nm (mean 205 nm) were reported for SiO_2 (Mcgeer *et al*. 2000; Ratte 1999).

TiO_2, SiO_2, and Al_2O_3 systems show similar peak shapes, which could indicate interactions of the different particle types in water. However, there are also significant differences in the sizing profiles of the resulting entities, indicating that processes may be occurring, which are specific to the composition of the ENP core.

Conclusions

To conclude, HDC-ICP-MS appears to offer a significantly improved approach for the characterisation of metal-based ENPs in complex environmental matrices because the chromatographic approach has a wide operational range and it is sufficiently robust to require no sample pre-treatment, even for samples as complex as lake water and sewage sludge supernatant.

ICP-MS (with collision cell technology) is highly element-specific, thereby allowing the simultaneous analysis of most of the commonly used nanoparticles in a single run and the inherent sensitivity of ICP-MS makes it suitable for detecting the low concentrations of nanoparticles that might be expected to be found in 'real' environmental samples (Boxall *et al.* 2007).

Sample analysis time is less than 10 minutes per sample, which makes it an appropriate methodology for the sizing and multi-elemental composition analysis of the large numbers of samples typically produced in environmental fate studies. This is a significant factor when compared to techniques such as field flow fractionation.

As hydrodynamic chromatography separates particles independent of their density, the gold nanoparticles can function as universal size calibration standards, without particle-specific size calibration being required. The gold particle standards could also have an extremely useful role if used as calibrants in other studies (analytical, toxicological, environmental fate, *etc*, allowing the production of easily comparable data. However, the quality of size calibration is essential to understand the behaviour and potential toxicological impact of ENPs in aquatic systems and although the use of gold nanoparticles helped to achieve very good data, more monodispersed calibrants, especially of the larger sizing standards (*e.g.* 250 nm), would be beneficial.

While the current approach is valuable for investigating changes in nanoparticle size characteristics within a system, the quantitative aspect of this methodology is still to be fully addressed, as quantitative ionic standards are not suitable for the column. Then again, post-column quantitative calibration highly underestimates the amount of particles present in the samples. As we cannot assume that particles of different sizes will give the same signal response (based on particle mass), it will be necessary to evaluate the method using well-characterised standards covering a wide range of sizes for each determinand of interest. Therefore well-characterised, monodispersed quantitative calibration standards have to be developed before the method can be used quantitatively.

It is believed that the analytical methodology described in this chapter will assist future research into the fate and impact of, and exposure to, ENPs in the environment and consumer products, particularly as a complimentary technique to *e.g.* field flow fractionation, or when combined with techniques which can provide information on particle shape, charge and coating.

Therefore, to validate this method for its use in environmental fate studies, it has been applied to assess the fate of inorganic ENPs (nanosilver as model nanoparticle) in activated sewage sludge processes (chapter 5).

Chapter 5

Fate of engineered silver nanoparticles in activated sludge systems

Introduction

The number of consumer products containing engineered nanoparticles (ENPs) is continuously on the increase. Currently, the most commonly used (based on product numbers) nanomaterial is nanosilver with 143 out of 606 commercially available, nanoparticle-based products (Woodrow Wilson International Centre for Scholars 2008). Nanosilver is currently used as an antibacterial agent *e.g.* in clothing, washing machines, wound dressings and cosmetic products. Many of these products will result in emissions to the sewage system (Benn and Westerhoff 2008) or to landfill sites so it is inevitable that nanosilver will be released to the environment. As the toxicity of ionic silver to organisms in the environment is well established (Mcgeer *et al.* 2000; Ratte 1999) and nanosilver is know to be particularly toxic to bacteria (Kim *et al.* 2007; Panacek *et al.* 2006; Morones *et al.* 2005; Yoon *et al.* 2007; Pal *et al.* 2007a) concerns have been raised over the potential adverse impacts of nanosilver on ecosystems.

While there are currently no measured data on concentrations of nanosilver in the environment, modelling investigations indicate that concentrations of nanosilver in the the environment, resulting from current use patterns, could range from 0.01-0.03 µg/L in surface waters and 0.02-0.43 µg/kg in soils (Mueller and Nowack 2008; Boxall *et al.* 2007). However, due to a lack of information on the fate of nanosilver in sewage treatment processes and the environment, these estimations are based on a

number of theoretical assumptions and provide no indication of whether the silver is in the dissolved form or present as free or aggregated particles. This means that it is very difficult to establish the risks, if any, to ecosystems.

This chapter describes a programme of work, using the HDC-ICP-MS method described in chapter 4, to explore the fate of nanosilver in sewage treatment systems in order to better characterise the risks of nanosilver in aquatic systems. Initial work focused on exploring the partitioning behaviour of nanosilver in activated sludge systems and on characterising the form of the nanosilver that is likely to be emitted from treatment works. These data were then used alongside simple exposure models to estimate likely aquatic exposure concentrations for nanosilver arising from a range of product types in use today. Finally, the exposure data were compared to reported effects data from nanosilver to determine whether a risk to the environment is likely.

Materials and methods

Test nanoparticles

A stock dispersion of silver nanoparticles (dispersed in ethylene glycol) was obtained from Sigma Aldrich (Poole, UK; Lot No.10109JH). The claimed particle size was 20-60 nm (TEM analysis), and the silver concentration was 12.84 wt % (by residue on ignition, manufacturer's data sheet. Prior to use, the total silver concentration in the stock was confirmed by ICP-MS. In addition, the size distribution of the nanoparticles was characterised by Dynamic Light Scattering (DLS; Mastersizer 2000, Malvern Instruments, UK), Transmission Electron Microscopy (TEM) and HDC-ICP-MS, and analysed for total silver by ICP-MS prior to use. The sizing methods are described in more detail below.

Test sludge

Mixed liquor sewage sludge (10 L) was collected from Broadholme Sewage Treatment Works (near Wellingborough, Northamptonshire, UK; figure 5.1) on the day of the experiment. The mixed liquor was kept aerated until used. The mixed

liquor suspended solids (MLSS) were determined immediately prior to the study and then adjusted, using tap water, to give a MLSS concentration of 2 g/L, this was then used in the subsequent adsorption study.

Figure 5.1. Mixed liquor at Broadholme Sewage Treatment Works.

Sludge adsorption study

Glass beakers (400 mL, acid washed) were prepared containing either 250 mL of the mixed liquor suspension or tap water. Analytical resources meant that it was only possible to explore three concentrations of silver nanoparticles. Therefore silver nanoparticles were spiked to the mixed liquor or tap water to give concentrations of 0.5, 5, or 10 mg/L. This concentration range was chosen so as to ensure that the levels of silver in the samples was detectable, even though it is expected that nanosilver will reach treatment plants in lower concentrations (Boxall *et al*. 2007). Each treatment concentration was prepared in triplicate. Three further vessels containing only MLSS were used as a control. The pH of the mixed liquor samples and the tap water was then determined, and found to be: 7 to 7.5, and 7.8 respectively.

Following addition of the nanoparticles, samples were shaken for six hours on a temperature-controlled shaking platform (20 °C). Samples were then taken straight from the tap water treatment, whereas in the MLSS treatment, the solids were allowed to settle for 30 minutes and the supernatant liquid was then removed. Tap water and supernatant samples were then subdivided for total silver analysis and size-based analysis using the methods described below. Samples for total silver analysis were preserved using formaldehyde.

Total silver analysis

For the analysis of the nanosilver stock dispersion, an aliquot (0.1 mL, which is equivalent to 0.12 g) was dissolved in nitric acid (2 mL of 2 % v/v) and then made up to volume (10 mL) with deionised water. 1 mL of this solution was pipetted into four test tubes and spiked with 40, 80, 120 µl of a 1 g/L silver ICP-MS standard solution (Aldrich, UK). This solution was then made up to 10 mL and further diluted (1:100 and again 1:125) with 2 % v/v nitric acid prior to analysis by ICP-MS.

The supernatant and tap water samples were diluted, as required, prior to analysis by ICP-MS (Agilent 7500CE, Cheshire, UK). Quantification was performed by standard addition, and, as additional quality assurance measures, aliquots of sample digests were spiked with 10, 20 or 40 µg/L of silver (Aldrich, UK; using an alternative source of stock solution from that used for the analyte additions) and measured along with the samples. The limit of detection was 0.7 µg/L.

Characterisation of nanoparticle size distributions

The particle size distribution of the silver ENPs in the stock solution, supernatant and water samples was determined using TEM and HDC-ICP-MS. TEM analysis was done on a JEOL 2010 microscope fitted with an energy dispersive x-ray spectrometer (EDX: Oxford Instruments, UK). The TEM was operated under standard conditions (200 kV acceleration voltage, 10-40 k magnification). Samples were prepared by applying ~ 20 µL of the sample to 200-mesh copper grids with carbon film (Agar scientific, UK). Following administration, the samples were allowed to dry before imaging. Image analysis was performed on at least ten representative images from

five different grids using Definiens Developer™, an automated object-based image analysis software (Tiede *et al.* 2008; Athelogou *et al.* 2007; chapter 4).

Supernatant and water samples were also analysed by HDC-ICP-MS. Size separation was achieved using a Polymer Laboratories hydroynamic chromatography column (size range 5-300 nm; Polymer Laboratories, Shropshire, UK) attached to an Agilent 1200 HPLC system with autosampler (Agilent, Cheshire, UK). Size calibration was achieved using gold colloids of mean diameters 5 nm (range 3.5-6.5 nm); 10 nm (range 8-12 nm); 20 nm (range 17-23 nm); 50 nm; 100 nm; 150 nm; 200 nm and 250 nm, were used (figure 5.2). The smaller particles (5-20 nm) were obtained from Sigma Aldrich (Dorset, UK) pre-dispersed in 0.01 % tannic acid with 0.04 % trisodium citrate, 0.26 mM potassium carbonate and 0.02 % sodium azide (the latter as a preservative). The larger particles were obtained from BBInternational (Cardiff, UK). Full details of the analytical method and calibration approach are described in chapter 4.

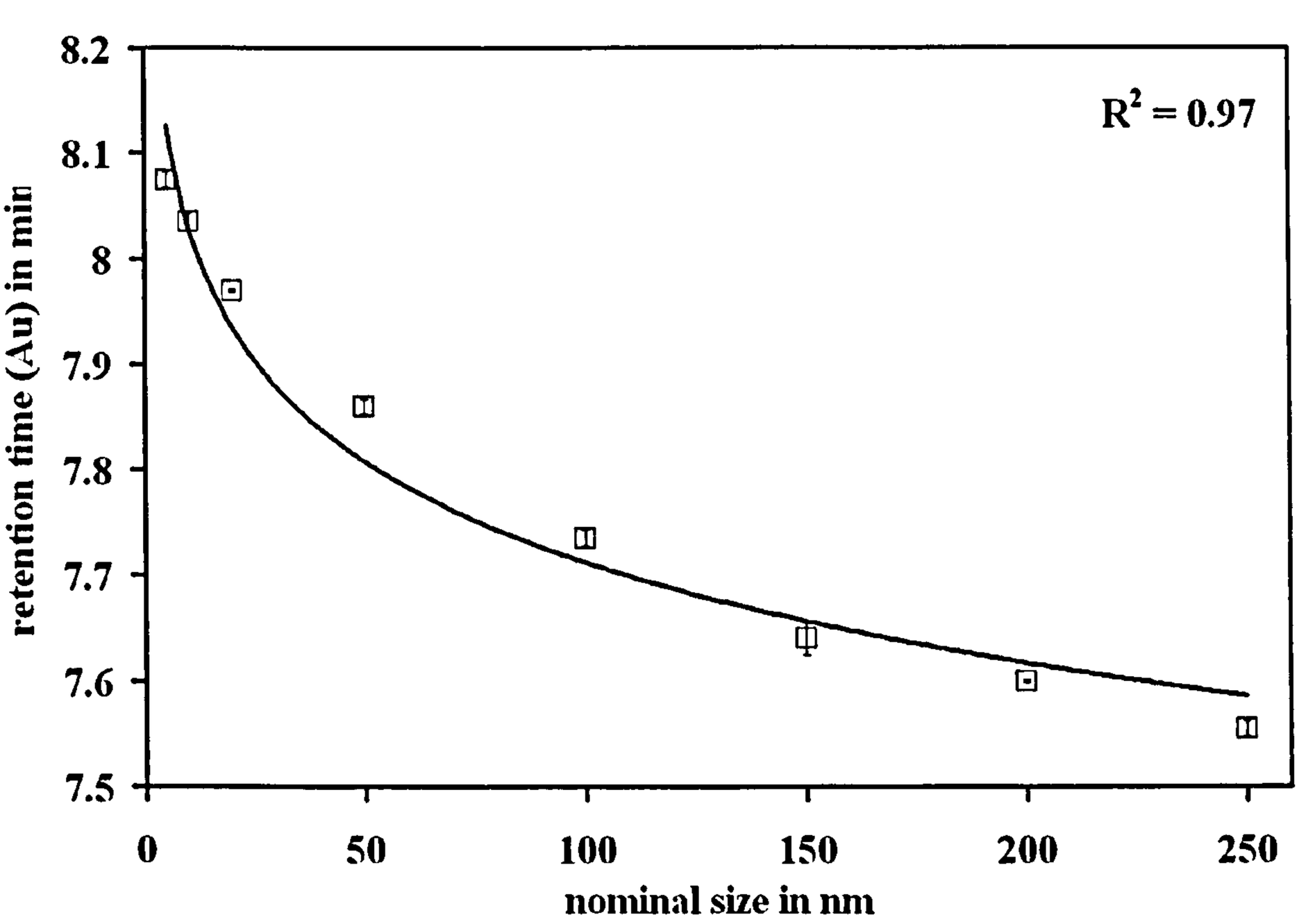


Figure 5.2. Size calibration of HDC column by Au nanoparticles. Error bars indicate standard deviation of the calibration runs prior and after sample analysis.

Data analysis

The total concentration of silver in the sludge phase was calculated, based on measured concentrations in the supernatant (corrected for background) using Equation 1.

$$Cs = \frac{At - Asu}{Wsl} \qquad \text{Equation 1.}$$

Where Cs = concentration in the sludge solids (mg/g), At = total amount of silver added (mg); Asu = amount of silver in the supernatant (mg); Wsl = amount of sludge solids in vessel (g).

Measured concentrations of silver in the supernatant were then plotted against estimated concentrations of silver in the sludge solids and a Freundlich isotherm was fitted to the data (Equation 2) using Microsoft Excel.

$$q = K_f c^{1/n} \qquad \text{Equation 2.}$$

Where q is the weight adsorbed per unit wt of adsorbent (mg/g); c is the equilibrium silver concentration in the liquid after the 6 h sewage sludge contact time (mg/L), K_f is the Freundlich coefficient and 1/n the Freundlich adsorption intensity parameter.

Results and discussion

Analysis of the stock dispersion showed the silver concentration to be 50.03 g/L, which is lower than the concentration claimed by the supplier and demonstrates the importance of characterising, supplied nanoparticles prior to testing. The average size of the nanosilver in the silver stock dispersion was determined to be 37.0 nm (n = 2, σ = 0) by HDC-ICP-MS (Figure 5.4a), 34.1 nm (n = 22, smallest particle = 21.9 nm, largest particle = 61.5 nm; table 5.1) by TEM and 68.1 nm by

DLS. The HDC-ICP-MS and TEM determined values were within close agreement and were within the size range of nanosilver claimed by the manufacturer. The sizing obtained by DLS was however significantly higher than the HDC-ICP-MS and TEM measurements and outside the manufacturers claimed size range. These differences might be explained by the fact that the silver ENPs in the sample were polydispersed or that interferences were present, both of which can result in inaccuracies when measurements are obtained using DLS (*e.g.* chapter 2 & 4, appendix 2).

Concentrations of silver in unspiked tap water and control supernatant from the sorption investigations were 0.001 mg/l (n = 3, σ = 0) and 0.02 mg/L (n = 3, σ = 0.03) respectively (table 5.1). Analysis of tap water spiked with silver nanoparticles, indicated that total silver concentrations at the end of the sorption study were within 16 % of the spiked nominal concentrations (table 5.1). Analysis of sludge supernatant from MLSS treatments indicated that a large fraction of the silver (92.7, 93.7 and 92.2 % for low, medium and high initial nanosilver concentrations respectively) had adsorbed to the sludge solids by the end of the study for all treatment levels.

Table 5.1. Averaged total amounts of silver (in mg/L) and silver recovery (in %) are shown for stock dispersion, tap water and sewage sludge supernatant samples analysed by ICP-MS (n = 3). Standard deviation is given in brackets.

	Blank	*Spike 1 (0.5 mg/L)*		*Spike 2 (5 mg/L)*		*Spike 3 (10 mg/L)*	
	Total Ag mg/L	*Total Ag mg/L*	*%*	*Total Ag mg/L*	*%*	*Total Ag mg/L*	*%*
Tap water	0.001 (σ = 0)	0.42 (σ = 0.08)	84.3	4.52 (σ = 0.05)	90.4	9.33 (σ = 0.50)	93.3
Sewage sludge supernatant	0.02 (σ = 0.03)	0.037 (σ = 0.009)	7.47	0.32 (σ = 0.027)	6.32	0.78 (σ = 0.045)	7.81

The Freundlich adsorption isotherm for the silver is shown in figure 5.3. Although, this is based on only three concentrations, the fit of the line was good indicating that the results provide a reasonable indication of behaviour over the concentration range tested. The Freundlich coefficient (K_f) was 5.48 (mg-Ag/g-MLSS) (L/mg-Ag) and

the average Freundlich adsorption intensity parameter (1/n) was 0.81 (unitless). Whereas K_f values for a comparable study on silver nanoparticles adsorption to biosolids arising from the washing of nanosilver-containing socks ranged from 3.40 to 16.88 (µg-Ag/g-biomass) (L/µg-Ag) and 1/n values from 0.62 to 0.71 for nanosilver and 16.88 (µg-Ag/g-biomass) (L/µg-Ag) and 0.66 for ionic silver (Benn and Westerhoff 2008). Studies into the partitioning of total silver in wastewater treatment works have reported Kd values of 56,000 - 1,390,000 L/kg (Shafer *et al.* 1998).

The precise mechanisms involved in the interactions between nanosilver and sewage sludge are not currently known. But possible reasons for the interactions include (1) the removal of nanosilver or the respective dissolved silver by sorption onto the solid sludge residue, (2) precipitation of dissolved silver in the form of silver chloride or sulphide, (3) nanosilver or dissolved silver interacting directly with organic matter, (4) particle aggregation and settling, and (5) mechanical/physical removal by particles entrapped within the mass of the residual solid material.

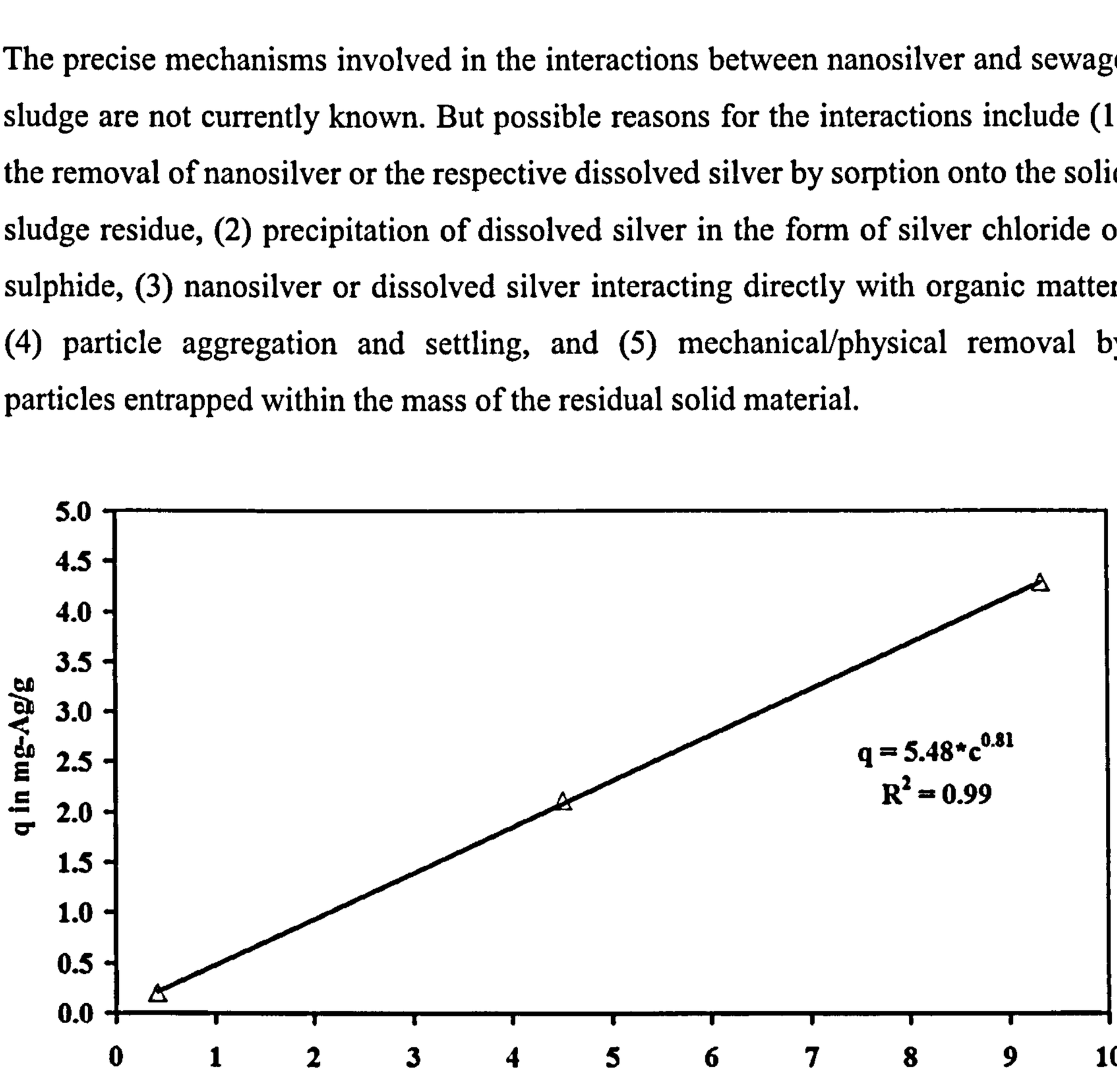


Figure 5.3. Adsorption isotherm for the sorption of nanosilver to sewage sludge. Initial Ag concentrations were 0.5, 5 and 10 mg/L, mixed liquor suspended solids were 2 g/L.

In addition to ENP concentration, properties such as the dynamics of dispersion, rate of dissolution, characteristics of the nanoparticle aggregates, surface area and surface characteristics are all likely to affect the behaviour and effects of engineered nanoparticles in environmental systems (SCENIHR 2007). Therefore when assessing environmental risks, it is not only be necessary to measure nanoparticle concentrations but also to develop an understanding of the morphology, size distribution and surface properties. Therefore, in addition to determination of total silver concentration, the samples (including the tap water quality control samples) were analysed by HDC-ICP-MS and TEM.

No silver-containing peaks were observed in HDC-ICP-MS chromatograms of either the unspiked sewage sludge supernatant or the tap water samples above the background (figure 5.4c). All the ENP-spiked tap water samples contained a single peak with an estimated average particle size of 38.3, 38.8, and 39.3 nm for low, medium, and high initial nanosilver concentrations respectively (figure 5.4b; table 5.2). These data indicate that the silver ENPs did not aggregate in water over the timeframe of the study. In contrast to the spiked water samples, HDC-ICP-MS chromatograms of supernatant from spiked MLSS (*e.g.* figure 5.4d, e and f) indicated that a mixture of sizes of particles in the range of < 5 to > 250 nm was present in the high, medium and low treatment levels. The most distinctive peak with the largest signal response in these corresponded to a size range of 2.97, 3.25 and 3.30 nm for low, medium and high initial nanosilver levels (obtained by extrapolation as retention time was outside the calibration range; table 5.2). The largest signal response in the mixture of less distinctive peaks corresponded to an average size of 52.0, 62.6 and 69.5 nm for low, medium and high initial nanosilver levels (table 5.2).

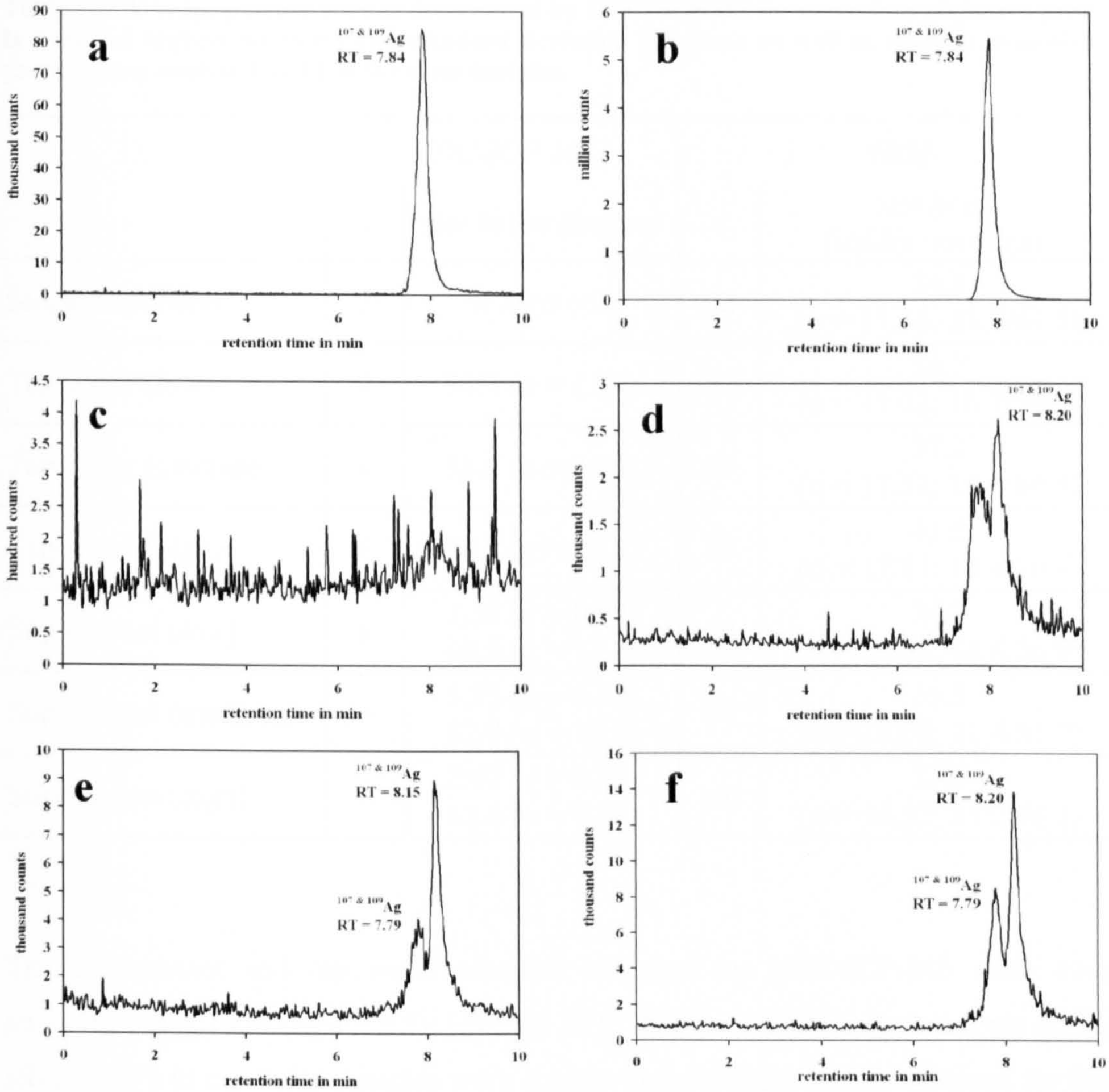


Figure 5.4. Representative chromatograms of (a) nanosilver stock dispersion, (b) nanosilver in tap water (c) unspiked sewage sludge supernatant (d, e, f) low, medium and high initial nanosilver levels in sludge supernatant samples obtained by HDC-ICP-MS monitoring isotopes $^{107\ \&\ 109}$Ag.

Table 5.2. Average particle sizes as determined by HDC-ICP-MS for nanosilver (first two peaks in order of highest intensity) and standard deviation are given as well as average nanosilver particle sizes analysed by TEM for same samples.

	HDC-ICP-MS		*TEM*	
	n	*size in nm (stddev)*	*n*	*size in nm (stddev; min/max)*
Stock dispersion	2	37.0 (σ = 0)	22	34.1 (σ = 11.56; 21.9/61.6)
Tap water (low)	6	38.3 (σ = 1.52)	21	34.7 (σ = 11.22; 16.7/54.8)
Tap water (medium)	6	38.8 (σ = 2.34)	60	37.2 (σ = 17.31; 15.8/89.8)
Tap water (high)	6	39.3 (σ = 2.15)	50	41.0 (σ = 17.11; 14.8/76.4)
Supernatant (low)	6	3.30 (σ = 0.94) 69.5 (σ = 17.6)	20	34.9 (σ = 12.33; 18.4/56.9)
Supernatant (medium)	6	3.25 (σ = 0.54) 52.0 (σ = 19.3)	34	35.5 (σ = 18.09; 11.4/67.7)
Supernatant (high)	6	2.97 (σ = 0.36) 62.6 (σ = 7.59)	102	33.5 (σ = 16.32; 13.2/78.5)

The supernatant and tap water samples analysed by HDC-ICP-MS were also analysed using TEM (figure 5.5). Like the HDC-ICP-MS, TEM measurements of the silver ENPs in tap water samples were similar to the measurements obtained for the stock dispersion. Measurements obtained for supernatant samples from spiked MLSS were also similar to the stock dispersion. This contrasts with the HDC-ICP-MS measurements, which indicated both, decreases and increases in silver particle size. The < 5 nm peaks obtained by HDC-ICP-MS for the sludge supernatant samples might indicate the presence either of small nanosilver particles, or dissolved silver *e.g.* interacting with natural organic matter, whereas the increase of particle sizes (~ 50 to 70 nm) could be due to particle/NOM interactions, which increase the effective (or hydrodynamic) diameter of the particle (Simon and Joner 2008; Hyung *et al.* 2007). Imaging of natural organic matter by TEM is limited and so is the spatial resolution (depending on instrument type); therefore, in this case, TEM image analysis did not reveal the differences of nanosilver behaviour in supernatant compared to tap water samples.

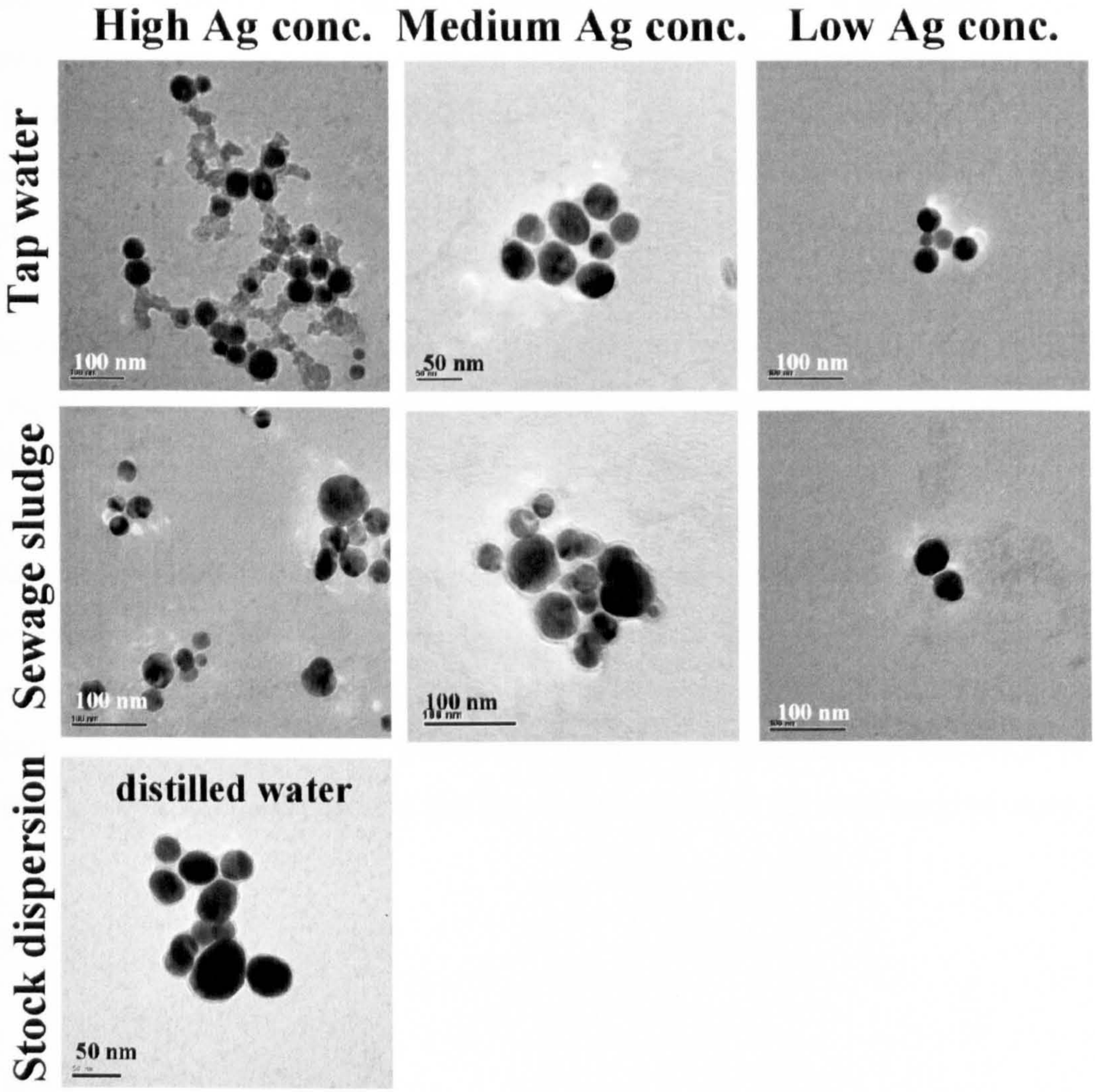


Figure 5.5. Representative TEM images of nanosilver particles in sewage sludge, tap water and of the nanosilver stock dispersion.

Overall the results indicate that a significant proportion of silver ENPs (> 90 %), if released to wastewater treatment systems, will associate with sludge solids. However, a silver fraction does remain in the aqueous phase and TEM and HDC-ICP-MS analysis demonstrate that a proportion of this will be in nanoparticle form. The data therefore indicate that engineered silver nanoparticles will be released to aquatic systems.

The data also provide a basis for estimating potential concentrations of silver ENPs in aquatic systems. A conservative estimate of concentrations of ENPs in receiving waters can be obtained using an adaptation of the surface water exposure algorithm developed by the Committee for Medicinal Products for Human Use (EMEA/CPMP 2003) for estimating concentrations of pharmaceutical substances in the environment. This algorithm (equation 1) estimates surface water concentrations based on the concentration of ENP in a product and the amount of product used per capita per day and removal in wastewater treatment. It assumes that the usage of a product is even over the year and that the sewage system is the main route of entry.

$$PEC_{sw} = \frac{C_{ENP}.U_{prod}.(1 - R_{stp}).F_{pen}}{WW_{inhab}.D} \qquad \text{Equation 3}$$

Where:

PEC_{sw} = predicted concentration in surface water (mg l^{-1})

C_{ENP} = concentration of engineered nanoparticle in product (mg g^{-1})

U_{prod} = daily usage of product (g capita^{-1} d^{-1})

R_{stp} = fraction of ENP removed during sewage treatment

F_{pen} = market penetration of nano-containing product

WW_{inhab} = amount of wastewater produced (l capita^{-1} d^{-1}) (default = 200)

D is the dilution factor in the receiving water (default = 10)

Data are available on the use of ENPs in a range of consumer products (Woodrow Wilson International Centre for Scholars 2008; Benn and Westerhoff 2008; Farkas *et al.* 2008; table 5.3). Silver ENPs may be used in a range of cosmetic and personal

care products, algaecides, dietary supplements, medicines, wound dressings, textiles, paints and washing machines, however, the available data has limitations *e.g.* in the Woodrow Wilson Inventory only products advertised on the internet are listed, and the Asian market is under represented due to language barriers. Also, the nanosilver concentration or the product emissions for listed products are not always available and difficult to estimate (*e.g.* surface disinfectants, wound dressings).

Based on the experimental data, 90 % ENP removal in treatment plants was assumed and PECs were calculated for 1 and 10 % market penetration of nanosilver products in each product category, where information was available on the concentration of the nanosilver in the product (0.24 and 2.38 ng/L for 1 and 10 % market penetration respectively). Table 5.3 shows the modelling results as well as the nanosilver concentrations in the product, the product and silver emissions per capita per day (estimated or known). While the predictions are based on a range of assumptions (*e.g.* they do not consider emissions from other product types; and they do not consider the potential for the nanosilver to accumulate in the environment), the data do provide a baseline against which to compare recently published ecotoxicity data on nanosilver.

A number of studies have investigated the effects of nanosilver on aquatic organisms, including bacteria, algae, invertebrates, fish and birds and a range of endpoints (Table 5.4). At this time the predicted environmental concentrations for nanosilver in surface waters (0.24 and 2.38 ng/L for 1 and 10 % market penetration respectively) may be of concern for aquatic organisms as these predictions are close to concentrations where effects were observed (10 ng/L Zebrafish embryos, Yeo and Kang 2008; table 5.4). In order to explore these risks further, much more work would be required, involving 1) more detailed sludge fate studies that not only characterise the sorption behaviour and size distribution of nanoparticles but which provide quantitative data on concentrations of different size ranges and species (including dissolved silver); 2) studies into the fate of silver nanoparticles in waters receiving effluent; and 3) more thorough ecotoxicity studies that determine the nature (including size distribution) of the exposure system and which attempt to determine the relative toxicity of different nanoparticle species.

In comparison to our exposure predictions, Benn and Westerhoff (2008) predicted a slightly higher effluent silver concentration of 0.01 µg/L, and wasted biosolids silver concentration of 2.8 mg-Ag/kg-biosolids assuming a common municipal treatment plant influent silver concentration of 5 µg/L. These estimated concentrations would probably also be too low to cause toxicity to aquatic organisms or bacteria.

Mueller and Nowack (2008) estimated silver concentrations (based on an estimated annual worldwide nanosilver production of 500 t/a) of 0.03-0.08 µg/L in water and 0.02-0.1 µg/kg in soil. Their calculated risk quotient (PEC/PNEC) for nanosilver was much smaller than one, and therefore adverse effects from nanosilver is not expected either. However, their PNEC calculation was solely based on two effects studies on bacteria (effects seen at 20 and 40 mg/L).

On the other hand, Blaser *et al.* 2008 predicted that in 2010, biocidal plastics and textiles will account for up to 15 % of the total silver released into water in the European Union. Despite the fact that only a limited risk assessment was possible at the time, their study indicated that PEC/PNEC ratios greater than one could not be ruled out for freshwater ecosystems, in particular sediments. However, they said that no risk is predicted for microbial communities in sewage treatment plants. Controversely, Choi and Hu (2008) found effects of nanosilver on nitrifying organisms (EC50 = 0.14 mg/L; table 5.4) and concluded that nanosilver reaching water treatment plants could have an impact on organisms in sewage sludge and therefore on the quality of the water treatment.

Table 5.3. Selected nanosilver containing consumer products, their nanosilver concentrations (highest estimates), product emissions and the resulting predicted nanosilver concentrations in surface waters (1 and 10 % market penetration).

Nanosilver containing products	*Source*	*Silver ENP concentration (mg/kg)*	*Product emission (g/capita/d)*	*Silver emission for product (µg/capita/d)*	*Predicted surface water concentration*	
					1 % (ng/L)	*10 % (ng/L)*
Cosmetics and personal care products	Woodrow Wilson Institute (2008)					
Cream		6	0.77	4.62	0.0023	0.023
Lipstick		6	0.015	0.9	0.00045	0.0045
Face mask		30	1.43	0.3	0.00015	0.0015
Shampoo		20	6.3	126	0.063	0.63
Toothpaste		20	1.34	26.8	0.013	0.13
Foods	Woodrow Wilson Institute (2008)					
Dietary supplement		10	15	0.15	0.000075	0.00075
Textiles	Benn & Westerhoff (2008)					
Textiles		10,000		290	0.15	1.45
Paints and coatings	Woodrow Wilson Institute (2008)					
Paint		2000	0.001	1.98	0.00099	0.0099
Appliances	Farkas *et al.* (2008)					
Washing machines				25	0.013	0.013
Total					***0.24***	***2.38***

Table 5.4. Effects of nanosilver on aquatic organisms.

Organism	*Endpoint*	*Concentration in mg/L (if not stated otherwise)*	*Reference*
Algae *Pseudokirchneriella subcapitata*	48h-LC50	0.19	Griffitt *et al.* 2008
Daphnids *Daphnia pulex*	48h-LC50	0.04	Griffitt *et al.* 2008
E. coli	OM disruption Accumulation of precursor OM-proteins	~43 & 86 ng/L	Lok *et al.* 2006
E. coli *V. cholera* *P. aeruginosa* *S. typhus*	Antibacterial effect	75	Morones *et al.* 2005
E.coli	Antibacterial activity; inhibition of growth rate	10 ng/L	Sondi and Salopek-Sondi 2004
E.coli	Inhibition of growth	1 – 12.5 µg	Pal *et al.* 2007b
Japanese quail *Coturnix coturnix japonica*	Effects on gut flora, in vitro: No significant changes in culturable enterobacteriaceae numbers in caecum Increase in numbers of culturable Gram-positive bacteria	25 mg/kg 5 mg/kg	Sawosz *et al.* 2007
Nitrifying organisms	EC50	0.14	Choi and Hu 2008
Zebrafish *Danio rerio*	48h-LC50	~7	Griffitt *et al.* 2008
Zebrafish embryos *Danio rerio*	Decrease in hatch rate Abnormalities and deformities	10 ng/L	Yeo and Kang 2008
Zebrafish embryos *Danio rerio*	Transport of particles into and out of embryos Abnormalities and deformities Concentration were only dead & deformed embryos were observed	~20 ng/L	Lee *et al.* 2007

Conclusions

Nanoparticles are now being used in a range of consumer products including costmectics, pharmaceuticals, food, textiles and washing implements. One of the main nanoparticles in use today is nanosilver. Following use, nanoparticles in consumer products may be released to the aquatic environment via sewage treatment works.

This study therefore explored the fate of nanosilver in activated sludge treatment. A combination of ICP-MS, HDC-ICP-MS and TEM was used to determine the sorption of nanosilver to activated sludge as well as to characterise the form of the silver in the sludge supernatant. Analysis of total silver concentrations demonstrated that a significant proportion of the nanosilver adsorbed to sludge. HDC-ICP-MS analysis demonstrated that a fraction of the silver that remained in the supernatant was in the nanoform, although due to the limitations of the method it was not possible to quantify this fraction. While the study was performed at concentrations much higher than concentrations that are likely to occur in wastewater treatment plants, they do provide an indication of how nanosilver may behave in real systems although further experiments would be beneficial to understand sorption at concentrations relevant to the real world.

Data from the sorption study was used alongside information on a range of nanosilver-containing products to estimate potential concentrations of nanosilver in the aquatic environment. While these predicted concentrations were low, they were close to effects data for bacteria and sublethal studies with fish. Due to the fact that the current study has only produced qualitative data on the presence of nanoparticles in sludge supernatants used and the published ecotoxicity data does not include detailed information on the nature of the exposure used, more work is required to fully characterise risks.

Further work into the fate and behaviour of nanosilver in the aquatic environment is therefore warranted. Moreover modelling and therefore risk assessment approaches

of nanosilver in the aquatic environment are so far highly limited. This is mostly due to the lack of data on current and future nanosilver production and usage, but also due to the lack of ecotoxicological data.

Chapter 6

Discussion: Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles

Introduction

As ENPs are expected to be used in a wide range of product types, it is likely that a range of environmental regulatory frameworks will apply to them. For example, industrial uses are likely to be covered by the REACH regulations (Regulation (EC) No. 1907/2006 2006), whereas applications in the pharmaceutical, biocides, veterinary medicines and plant protection products will be covered by other specific frameworks (*e.g.* Aitken *et al.* 2006). However, at present, regulations specifically developed for ENPs do not exist anywhere in the world.

The environmental safety data requirements for different regulatory schemes vary, but typically information needs to be generated on: 1) environmental fate properties (including degradation, environmental distribution, bioaccumulation and secondary poisoning); 2) environmental hazard (including hazard to organisms in the aquatic (also sediment), terrestrial and atmospheric compartments and microbes in sewage treatment systems); 3) environmental exposure; and 4) environmental risk for different compartments. Guidance is available on how to perform these studies and risk assessments in relation to conventional chemicals (*e.g.* Aitken *et al.* 2006; EMEA/CPMP 2003; EMEA/CVMP 1997) However, the suitability of most of the current environmental testing schemes, methods and models for ENP assessment has been questioned (Warheit 2008; Hansen *et al.* 2007a).

Major questions, specific to environmental testing of ENPs, include: What material should be tested and which form should be studied (*e.g.* coated or not)? What matrix and ENP exposure levels should be studied? How should the ENPs be introduced to a study system? What parameters should be monitored during a study? How should the ENP test material and media be characterised before, during, and after testing? Therefore this chapter draws upon the experiences made in chapters 2 to 5 and on existing knowledge on the characteristics, detection, fate, effects, and exposure of ENPs in environmental systems in order to develop recommendations on the design of environmental fate and effects tests for ENPs. The chapter focuses mainly on detection, characterisation and testing in aqueous systems in laboratory-based fate and ecotoxicity studies. Gaps in the current knowledge related to environmental risk assessment are also identified and recommendations are provided on future work to address these gaps.

Environmental testing of engineered nanoparticles

Selection of test ENPs

A nanoparticle can consist of a compound or an element; it can be colloidal or in powder form, coated or stabilised (*e.g.* designed for surface functionality) and can have different crystal structures and shapes (Nowack and Bucheli 2007). Stabilisation of a particle to prevent aggregation and loss of functionality can be achieved by giving it a negative or positive charge (electrostatic repulsion) by decreasing or increasing respectively the pH well below or above the point of zero charge (Waychunas 2001). ENPs may also be stabilised sterically through the adsorption or covalent or ionic binding of macromolecules to the particle surface (Lourenco *et al.* 1996).

The environmental fate and ecotoxicity of ENPs may be influenced by a number of properties, including: particles size and size distribution, solubility and state of aggregation, elemental composition, mass and number concentrations, shape and crystal structure, surface area, charge and chemistry and the presence of impurities (chapter 2). As a result, ENPs of the same material but with different crystal

structure, surface coating, shape or size can have very different behaviour, uptake and effects (*e.g.* Chithrani *et al.* 2006; Warheit *et al.* 2007; Morones and Frey 2007; see gold stabilisation results presented in chapter 4). For example, the kinetics and saturation concentrations of gold ENPs in cellular uptake studies have been found to be highly dependent on the physical dimensions of the test particles (Chithrani *et al.* 2006). Triangular shaped Ag particles have been shown to have a higher antibacterial activity than spherical or rod shaped ones (Pal *et al.* 2007a), antibacterial activity has also been shown to be dependent on particle size (Morones and Frey 2007). The behaviour and toxicity of TiO_2 ENPs has been shown to be affected by the crystal structure and the presence and nature of surface coatings (Warheit *et al.* 2007). Lyon *et al.* (2006) demonstrated antibacterial activities of differently prepared and sized fullerene water suspensions (Lyon *et al.* 2006). Finally, the degree and kind of agglomeration has been shown to affect carbon nanotube toxicity (Wick *et al.* 2007). As ENPs made of the same material can behave so differently (Limbach *et al.* 2005), it is essential that fate and effects studies, performed as part of the environmental risk assessment of an ENP in a product, consider the actual form of the ENP that is being introduced into the product. It may also be appropriate to address the effect of any manipulation of the ENP (*e.g.* functionalisation, stabilisation etc.) that might be performed during the production process, for example if an ENP particle is chemically manipulated prior to inclusion in a product, it is probably more appropriate to assess the manipulated particle. Finally, it is important to recognise that, once released into the environment, coatings on an ENP surface may degrade or change over time so it may also be necessary to consider the fate and effects of the altered ENP. This is analogous to pesticide environmental risk assessment where in some cases, the environmental fate and effects of an environmental transformation product of the parent pesticide needs to be explored rather than the parent compound. Whatever test material is selected, prior to testing, the ENPs tested should be extensively characterised in terms of particle size, size distribution, shape, surface area (including bioavailable surface area), redox potential and properties, purity and identity of contaminants, catalytic activity, dissolution potential, potential to generate reactive oxygen or nitrogen species and agglomeration state (Balbus *et al.* 2007).

Analytical methods for determining many of these characteristics are described later in this chapter.

Selection of test concentrations

Following release to the environmental systems, most ENPs will aggregate to some degree (*e.g.* Fortner *et al.* 2005; Phenrat *et al.* 2007; Dunphy *et al.* 2006). Aggregates may then settle out (Brant *et al.* 2005b; Teeguarden *et al.* 2007). The degree and kinetics of aggregation and the size range of the aggregates is dependent on the characteristics of the particle, and the characteristics of the environmental system (which is discussed below) and the concentration of the particle (Phenrat *et al.* 2007; Dunphy *et al.* 2006; Hyung *et al.* 2007). As aggregation behaviour is likely to affect fate and toxicity, it is important that at least some of the test concentrations used in environmental fate and ecotoxicity experiments are environmentally relevant (Hansen *et al.* 2007b). For example, unlike traditional contaminants, it is possible that higher toxicity could be observed at lower test concentrations as the extent of aggregation at these concentrations is likely to be reduced and more of the ENP is in the free particulate (unaggregated) form.

In the absence of environmental monitoring data, one approach to assess the potential levels of ENPs released into the environment and hence levels for testing is to use environmental exposure models. For example, Boxall *et al.* (2007) applied simple algorithms to estimate the potential concentrations of a range of ENPs arising from consumer products. Predicted concentrations, obtained using these models (table 6.1), are significantly lower than the concentrations that are typically being used in current ecotoxicity investigations which means that the results of many of the currently published studies may not represent impacts in the real environment (see also chapter 5 for a general description of the principles underlying the algorithms used).

Table 6.1. Predicted concentrations of ENPs arising from use in consumer products (Boxall *et al.* 2007).

	Water (µg/l)	*Soil (µg/kg)*
Ag	0.010 0.0024 (chapter 5)	0.43
Al_2O_3	0.0002	0.01
Au	0.14	5.99
CeO_2	<0.0001	<0.01
fullerenes	0.31	13.1
hydroxyapatite	10.1	422
latex	103	4307
organo-silica	0.0005	0.02
SiO_2	0.0007	0.03
TiO_2	24.5	1030
ZnO	76	3194

Selection of test conditions

The experimental conditions and the test medium will significantly influence the form of the ENP. Aggregation, stabilisation, and dissolution as well as the ecotoxicity of ENPs differ greatly across aquatic systems. For example, changes in ionic strength can cause particle destabilisation and aggregation of ENPs (Waychunas 2001; Stolpe and Hassellöv 2007; Guzman *et al.* 2006a). The presence of humic substances can contribute to nanoparticle steric and electrostatic stabilisation of ENPs (Hyung *et al.* 2007; Illes and Tombacz 2006; Pelley and Tufenkij 2008). Alterations in pH of the test media can influence the surface potential and thus may influence the stability, aggregation and transport of nanoparticles. Any pH changes during fate and ecotoxicity experiments, especially close to the point of zero charge, may therefore have profound effects on the results (Degen and Kosec 2000; Guzman *et al.* 2006b). Different systems (*e.g.* sea water, fresh water, buffered systems) featuring different temperature, pH, and ionic strength as well as organic complexation can also affect the solubility of materials and thus

the particle dissolution kinetics (*e.g.* Chen and Elimelech 2007; Millero *et al.* 2001; Sholkovitz and Copland 1981; Spokes *et al.* 1996; Adams *et al.* 2006).

Hence, the type of matrix to be used in an environmental fate or ecotoxicity experiment requires careful consideration. As we do not yet have a detailed understanding of those factors affecting the characteristics of ENPs in natural systems, it may be appropriate to explore the fate and effects of a study ENP in a range of matrices selected to represent the range of physico-chemical parameters that occur in the natural environment. Environmental pH values generally range between 6 (4 in bogs) and 9 in freshwaters, and 8 and 9 in seawater, while ionic strength varies between 10^{-4} and 10^{-3} mol L^{-1} in freshwater and is relatively constant at 0.7 mol L^{-1} in seawater (Andrews *et al.* 2004; Gibbs 1970; Stumm and Morgan 1996). DOC concentrations, which contribute the major fraction to natural organic matter in aquatic systems, may reach up to 8×10^{-4} mol L^{-1} in freshwater, but usually do not exceed $(6 - 9) \times 10^{-5}$ mol L^{-1} in the ocean (Hansell and Carlson 2002). By selecting test matrices for fate studies and ecotoxicity investigations that provide a broad coverage of these parameter ranges, it is likely that a better understanding of the distribution and effects of ENPs in real environmental systems will be obtained. Such data will also be invaluable in building our understanding of the important factors and processes affecting ENP environmental risks and could ultimately be used to generate modelling approaches to extrapolate across different environmental conditions.

For some ENPs, the light conditions employed in an experimental study may also be important, for example TiO_2 ENPs have been suspected to be of great photochemical importance due to potential oxygen radical formation, which in turn may not only have toxicological significance for water borne biota (Adams *et al.* 2006), but can also effect the water chemistry and redox cycling of metals.

For many ENPs, particularly those that aggregate, exposure in sediments may be more important than exposure in the water column. In these instances, it may be more appropriate to perform studies into the effects of the ENP on benthic organisms rather than water-dwelling species.

Careful consideration should also be given to the selection of the type of test vessel used in the experiments. For example, while Teflon generally finds application in

trace metal research due to the low adherence of any materials to its surface and its low metal content in the material after production processes, our studies have demonstrated strong adherence of ZnO ENP dispersions to the walls of Teflon containers. This was not the case with ordinary boro-silicate glass. Unpublished soil sorption experiments in our laboratory, using gold colloid nanoparticles and ICP-MS analysis, also demonstrated that a significant proportion of the particles adsorbed to Teflon and plastic containers, whereas less loss was observed in glass. We would therefore recommend that the interactions of a study ENP with all materials employed in any planned fate and effects studies are determined before the study commences and that measures are taken to reduce these interactions where possible *e.g.* through the selection of alternative test vessel materials or pre-conditioning of test vessels.

Introduction of ENP to test system

The introduction of a study ENP into the test system can be challenging. Agglomeration and aggregation are common problems when preparing dispersions for testing from dry material (Adams *et al.* 2006) Further complications can arise when stock dispersions are diluted as this can lead to aggregate formation due to low surfactant concentrations in the final solution (Franklin *et al.* 2007). A range of approaches have therefore been proposed for preparing test items including the use of surfactants and solvents, sonication, filtration and stirring for prolonged periods and pH manipulation (*e.g.* Hyung *et al.* 2007). Surfactants such as sodium dodecyl sulfate (SDS) and solvents such as tetrahydrofluran (THF) have been proposed to disperse metal ENPs and fullerenes, however, their application for environmental effect studies is questionable (Handy 2008). Sonication can be applied following dispersion to reduce particle aggregation but this may only momentarily de-agglomerate the particles.

The selection of method of preparation will depend on the objectives of the study, for example if the aim is to understand relationships between particle properties (*e.g.* size) and environmental behaviour or effects, it is probably appropriate to take measures to ensure that the particles are in the free un-aggregated form, where possible. However, when testing a particle for product risk assessment purposes, it

may not be appropriate to take these measures as such manipulated exposures are highly unlikely to occur in natural systems. It is the chemical conditions of the media, favourable to agglomeration or not, that will determine the kinetics of agglomeration, as for example observed for ZnO (Franklin *et al.* 2007; Zhu *et al.* 2008). Once a study is running, the size distribution may not be stable and over time most ENPs will eventually re-aggregate. Following the initiation of a test it may be appropriate to agitate the test medium during the experiment in order to mimic physical disturbances that are likely to occur in natural systems and which could affect the aggregation state of a study ENP.

Monitoring of test and exposure conditions

As described above, the fate, behaviour and effects of ENPs will be highly dependent on the characteristics of the ENP, the environmental conditions and the ENP concentrations. Therefore, in order to interpret the results of environmental studies with ENPs, it is important that not only the ENP exposure concentration but also selected characteristics of the material (*e.g.* size distribution and fraction of ENP in the dissolved form) are measured during the fate and effects studies.

The size distribution should be monitored over the course of the experiment to clarify whether the free nanoparticles, aggregated particles, solutes or a mixture of these are being studied. In order to clearly assign the ecotoxicity of a substance to its nanoparticle form, it may be appropriate to compare the effects of the ENPs with the effects of the corresponding bulk material and resulting ions in solution. This approach has previously been applied in studies addressing toxicity of ZnO ENPs on Zebrafish (Zhu *et al.* 2008; Griffitt *et al.* 2007) and on freshwater microalgae (Franklin *et al.* 2007).

Important environmental parameters such as pH, ionic strength and DOC should also be regularly monitored throughout the fate and effects studies. Detailed characterisation and monitoring may overcome many of the problems associated with comparing the results of different studies with ENPs (*e.g.* Griffitt *et al.* 2007). Approaches for analysis and characterisation are discussed below.

Analysis and characterisation of ENPs in environmental studies

General considerations on analytical requirements and limitations

Considering the many factors possibly influencing nanoparticle – environment interactions, analytical techniques addressing a wide range of nanoparticle properties are needed to measure the key parameters affecting ENP toxicity and behaviour. A wide range of methods is available for detection and characterisation, including microscopy-based methods, centrifugation, dynamic light scattering, voltammetry and size separation methods coupled to analytical instruments (chapter 2; table 6.2).

The selection of the analytical method will depend on the question being asked. For example, when characterising a test ENP prior to testing, it is likely that a wide range of characteristics will need to be measured (see earlier) so a number of the methods described in table 6.2 will be necessary. At this stage, as large amounts of the test item will be typically available and as interferences will be minimal, the characterisation will be less challenging than for samples obtained from during environmental fate and ecotoxicity studies.

For less complex environmental studies (*e.g.* water-only ecotoxicity studies and studies into aggregation in water), it may be possible to use some of the more simple and rapid analytical approaches described in table 6.2 such as DLS and centrifugation to generate data on the ENP characteristics in a test over time. Microscopy-based analysis of samples from these studies is also possible. However, when working in more complex systems (*e.g.* sorption and persistence studies in sludge, soils and sediment/water systems; water-sediment ecotoxicity studies; or bioavailability studies), the analysis becomes significantly more challenging and techniques such as DLS, and electron microscopy can struggle to distinguish between the test ENP and naturally occurring nanomaterials as well as between the dissolved and particulate form (appendix 2; Banfield and Zhang 2001). Also, DLS results (*e.g.* more polydisperse samples) should be treated with some caution as shown by the TEM, HDC-ICP-MS and DLS results comparison in chapter 5.

Ideally in these studies, the analytical method should not only cover the defined size of ENPs up to 100 nm, but should also allow the measurement of ENP aggregates above 100 nm. In these instances more sophisticated separation methods such as FFF

and HDC coupled to detection by mass spectrometry are therefore appropriate (table 6.2). As engineered nanoparticles are expected to occur in low particle number concentrations in the environment, if fate and ecotoxicity studies are being performed at more environmentally relevant concentrations, sensitive methods providing low limits of detection or high spatial resolution (in the case of visual techniques, *e.g.* electron microscopes) will be required.

Many of the techniques summarised in table 6.2 are destructive and do not allow further examination of the exact same sample for comparison and validation on a single particle basis, especially those for which sample preparation is required. This can in some cases lead to sample alteration (*e.g.* drying artefacts, dissolution, and aggregation) and severely affect results as well as their comparability and reproducibility. For example, electron microscopy based methods have to be operated under vacuum conditions and therefore require dry samples. Figure 6.1 shows transmission electron microscopy (TEM) images of 50 nm aminodextran stabilised gold colloids (BBI, Cardiff/UK) dispersed in a suspension of 10 mg/L Standard Suwannee River natural organic matter (NOM). A drop of this suspension was applied to a copper grid with carbon film and allowed to dry prior to imaging under standard conditions. Images in the first row suggest strong interactions of gold colloids and NOM. However, second row images show gold colloids not attached to NOM suggesting the possibility of drying artefacts (figure 6.1).

Table 6.2 Most common analytical techniques for environmental testing of ENPs: requirements and application range.

Analytical tool	*Parameter*	*Particle type*	*Sample requirements*	*LOD, spatial resolution, separation range*	*Comments*
Atomic force microscopy	Visualisation State of aggregation Shape	All types	Dry and liquid samples Single particle	~ 5 nm to several µm	Chemical force microscopy for element analysis
N_2 adsorption, BET	Specific surface area Porosity	Powders	Solid sample	~ 5 nm to several µm	
Centrifugation	Size fractionation	All types	Liquid sample Whole sample		Possible effects such as diffusion gradient
Dynamic light scattering	Size distribution	All types	Liquid samples Monodispersed	5 nm to several µm	No element analysis available Not suitable for polydispersed samples
Electron microscopy	Visualisation State of aggregation Shape	Elements with high electron density (*e.g.* metals)	Dry sample Single particle	~ 0.3 nm to several µm	In combination with EDX, TEM: EELS for element analysis, SAED mode etc.
Electrophoretic mobility	Zeta potential	All types	Liquid sample Whole sample		pH dependent surface charge Aggregation behaviour
Environmental scanning electron microscopy	Visualisation State of aggregation Shape	Preferably elements with high electron density (*e.g.* metals)	Humid samples Single particle	1 – 50 Torr Up to 100 % humidity	In combination with EDX

Table 6.2. ***Continued.***

Analytical tool	*Parameter*	*Particle type*	*Sample requirements*	*LOD, spatial resolution, separation range*	*Comments*
Field flow fractionation	Size distribution	All types	Liquid samples Polydispersed Complex media	1 nm to several µm	In combination with UV/Vis, ICP-MS, MALLS, DLS etc.
Filtration	Size fractionation	All types	Liquid sample Whole sample	Down to 1kDa	Possible limiting effects such as charging, clogging etc.
Hydrodynamic chromatography	Size distribution	All types	Liquid samples Polydispersed Complex media	5 - 1200 nm	In combination with UV/Vis, ICP-MS etc.
Size exclusion chromatography	Size distribution	All types	Liquid samples Polydispersed	Size range dependent on pore size	Solid phase interactions Good peak resolution
Voltammetry	Free metal ions Total substance concentrations Metal speciation	Especially metals	Liquid samples	ng range	Potential interferences from ENPs during ion analysis need to be evaluated Highly sensitive
Diffusive gradients in thin films (DGT)	Free metal ions Metal speciation	Metals	Liquid samples	Sensitivity depends on exposure time	Exposure time up to weeks required for in-situ environmental tests, but suitable for monitoring
X-ray spectroscopy, scattering	Surface chemical & structure analysis	Powders	Solid samples, powders	µg to mg	Poor spatial resolution Non-destructive

Analytical developments focusing on natural matrices (*e.g. in situ* analysis, reference materials & differentiation) as described above are essential for progress in environmental testing. However, additionally methods to understand ecotoxicity mechanisms are needed (Maynard *et al.* 2006). Powers *et al.* (2006) stated very clearly that: "Key parameters affecting biological activity of nanoparticles are largely unknown at this point; characterisation of test material must be comprehensive and broad in scope. A study conducted with material that has not been characterised with respect to a property later found to be critical for toxicity will ultimately be of little value" (Powers *et al.* 2006: p. 1, Introduction). It is also essential to characterise and monitor changes in the test items during tests as nanoparticles strongly react on change of environment (see earlier). For example, particles added to the test medium will most certainly show changes such as aggregation or dissolution. Here, time is a major issue and *in situ* techniques are needed for providing fast analysis without disturbing the experimental set-up. Sample storage is not recommended as this could cause further artefacts. The lack of reference materials for environmental testing as well as analytical standards (*e.g.* size standards) complicates nanoparticle research and means that method validation is problematic and that different studies are not comparable (Maynard *et al.* 2006; Brunner *et al.* 2006).

Different nanoparticle parameters often have to be analysed by different analytical methods. Prior to the characterisation of samples, it is important to know, not only what kind of information a specific technique can provide (*e.g.* size distribution, elemental information, sensitivity, structural information etc), but also the requirements of the sample (dry, pure media, size range, elemental composition etc.) for each method to make analysis possible and to guarantee meaningful results (table 6.2). For instance, if a stock dispersion of nanoparticles is analysed by dynamic light scattering, this will provide the scattering intensity weighted diffusion coefficient (or hydrodynamic diameter), which provides good results for a monodispersed sample. However, once the sample is not monodispersed *e.g.* other particles (dust, sediment, food, algae) are present as is mainly the case in environmental samples, dynamic light scattering does not provide valuable data any longer, as it cannot distinguish between different types of particles (*e.g.* elemental characterisation) and fails to give precise sizing data for polydispersed samples (see chapter 5 for an example).

Universal instruments that can track the release, concentration and transformation of engineered nanomaterials in air, water, and soil still have to be developed (Maynard *et al.* 2006). To date, no single protocol exists to characterise the fate and behaviour of nanoparticles *in situ*, or in general in complex media such as water, sediment and soil. Separating a wide range of particle sizes (~ 1 – 1000 nm) in chemically diverse, polydispersed samples at low concentrations is a major challenge. Minimal sample preparation is desirable, but potential artefacts during analysis are difficult to assess and may be unavoidable. HDC-ICP-MS may be one possible solution to this problem.

Overview of existing techniques

Chapter 2 and appendix 2 provide detailed and full overviews of analytical techniques possibly suitable for nanoparticle analysis in complex (*e.g.* environmental and food) media. In this section, not to be repetitive, solely a brief overview of existing techniques will be provided.

In general and considering individual situations, the following methods are potentially suitable for nanoparticle characterisation in environmental studies:

Microscopy-based techniques capable of visualising nanoparticles include the well-known scanning and transmission electron microscopy (SEM and TEM) and atomic force microscopy (AFM) of the group of scanning probe microscopes. Apart from providing information on particle aggregation, dispersion, size, structure and shape, for additional elemental information can be obtained by coupling EMs to energy-dispersive spectrometry (EDS) or to electron energy loss spectrometry (EELS, available for TEM) (Mavrocordatos *et al.* 2004). The new development of chemical force microscopy allows elemental analysis via probe-sample interactions (Sugimoto *et al.* 2007). Whereas liquid samples can be analysed by AFM under certain conditions (Balnois *et al.* 2007), the obligatory vacuum chamber in electron microscopes prevents imaging of liquid or wet samples. To overcome sample alteration and imaging artefacts caused by sample preparation, *e.g.* drying (figure 6.1), environmental scanning electron microscopy (ESEM), wet scanning transmission electron microscopy (WetSTEM) and wet scanning electron microscopy (WetSEM™) as well as X-ray microscopy (XRM) can be of advantage

(Thieme *et al.* 2007; Doucet *et al.* 2005a; Bogner *et al.* 2005; Timp *et al.* 2007). Microscopy analysis is a highly valuable tool for nanoparticle characterisation; however, it is prone to imaging artefacts and the small amount of sample imaged and analysed makes interpretation and statistical validation difficult.

Techniques for size distribution analysis of polydispersed samples include field flow fractionation (FFF), hydrodynamic chromatography (HDC) and size exclusion chromatography (SEC). They can be hyphenated to several different types of detectors like ICP-MS, UV-visible spectroscopy, nephelometry, static light scattering (SLS) and can find more and more applications due to their wide size separation ranges and relatively mild sample perturbation (Helfrich *et al.* 2006; Yegin and Lamprecht 2006; Stolpe *et al.* 2005). While SEC has higher separation efficiency, it can suffer from stationary phase interactions. HDC shows comparably poor peak resolution but avoids the phase interactions. FFF can be complex and time-consuming, but may yield good resolution when the detection window is limited to 1-2 orders of magnitude.

For nanoparticle separation or fractionation, techniques like ultracentrifugation, nanofiltration, and cross-flow filtration are commonly used (Lead and Wilkinson 2006; Liu and Lead 2006; Tang *et al.* 2008). Nanoparticle solubility is only quite recently considered in ecotoxicological studies and for the separation of nanoparticles from their solutes filtration, dialysis and centrifugation have been applied (Franklin *et al.* 2007; Zhu *et al.* 2008; Griffitt *et al.* 2007). However, centrifugation can suffer from a size gradient and filtration not only depends on pore sizes, but issues such as charging effects and clogging have to be considered, whereas dialysis is highly time-consuming. Therefore, analytical techniques have to be established especially for environmental testing to clearly separate not only the nanoparticle form from bigger counterparts, but also to distinguish between their solute forms. Here options could involve voltammetry, diffusion gradients in thin films (DGT), diffusion equilibration in thin films, and the permeation liquid membrane method (PLM) (Buffle and Tercier-Waeber 2005; Salaun *et al.* 2004; Slaveykova *et al.* 2004; Gimpel *et al.* 2003; Motelica-Heino *et al.* 2003; Zhang *et al.* 1999; Tercier-Waeber *et al.* 1998).

Also applicable for determining particle size distributions are particle counters (Ito *et al.* 2003), dynamic and static light scattering (DLS, SLS). DLS features fast and *in situ* sizing (Huve *et al.* 1994), but as mentioned previously, it shows considerable limitations with regard to polydispersed samples or in complex media (Bootz *et al.* 2004). Spectroscopy-based methods of potential value include laser-induced breakdown diffraction (LIBD) (Bundschuh *et al.* 2001b), nuclear magnetic resonance (NMR), and UV-Vis spectroscopy (Pesika *et al.* 2003).

Analysis of surface area can be achieved by the Brunauer Emmett Teller (BET) method or by calculation using particle diameters (obtained by *e.g.* AFM, TEM). The determination of the surface charge in dependence of pH (by electrophoretic mobility, zeta potential) can provide information on *e.g.* particle aggregation or particle behaviour during filtration.

X-ray-based methods such as X-ray absorption (XAS), fluorescence (XRF), and photoelectron spectroscopy (XPS) as well as diffraction (XRD) are in general highly surface-specific and can provide information on surface properties and coatings, crystallographic structure or elemental composition (Nurmi *et al.* 2005).

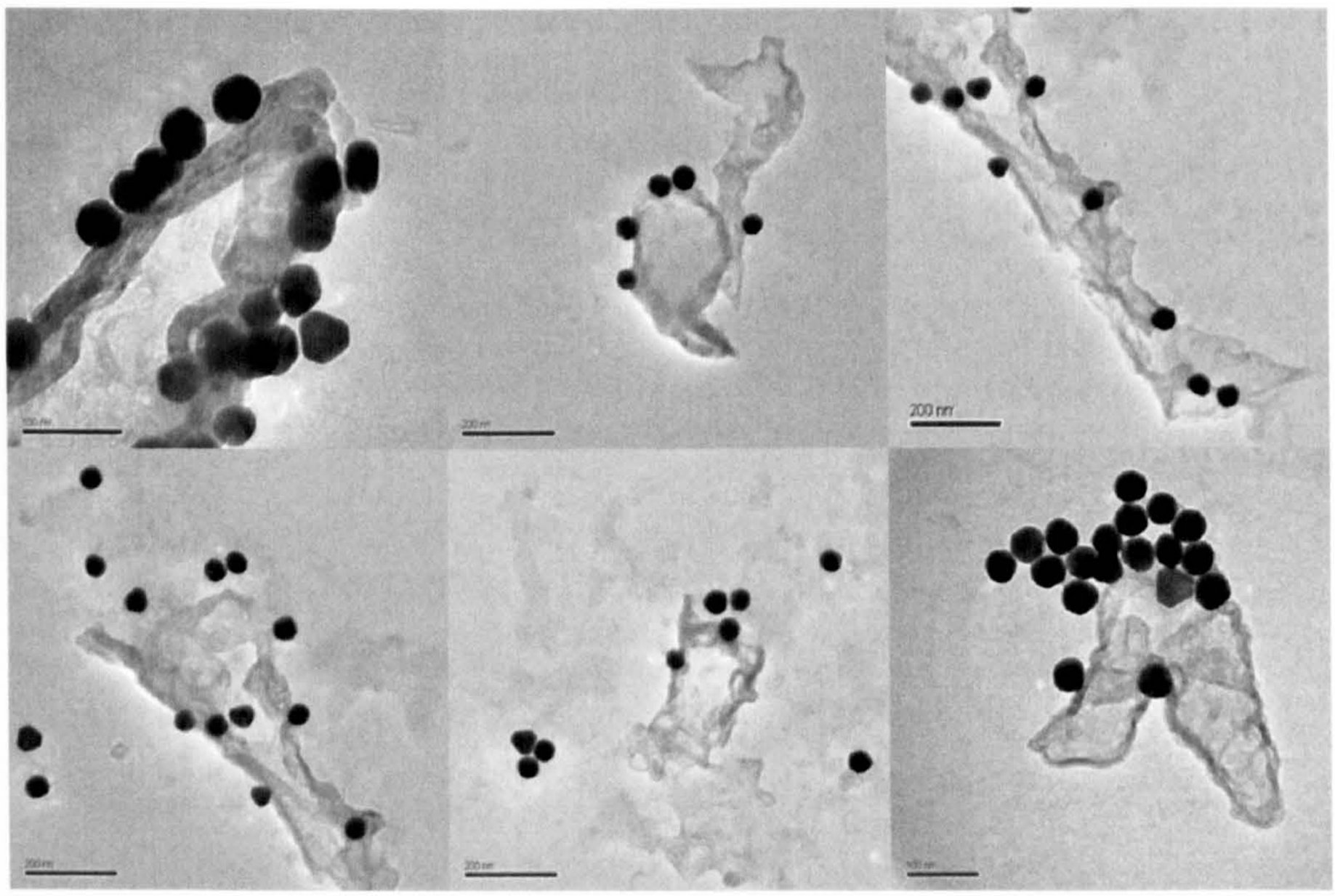


Figure 6.1. TEM images of 50 nm aminodextran stabilised gold colloids in a suspension of 10 mg/L NOM (Standard Suwannee, USA) and in distilled H_2O.

Conclusions and research needs

To summarise, there are a large number of challenges associated with the environmental testing of ENPs compared to substances, which have traditionally been tested. In this chapter, we have attempted to highlight the major challenges and to present possible solutions to these. However, this science is currently in its infancy and environmental testing of engineered nanoparticles urgently requires the development of testing guidelines to allow for the comparison and interpretation of data from environmental studies. In order to achieve more comparable and reproducible data, standards have to be agreed upon and reference materials need to be developed. In the meantime, we recommend that applied methods (experimental set-up, sample preparation, and analysis) in ongoing and future studies are fully documented to enhance transparency and comparability of obtained data. We further encourage open discussion of "negative data", which would be of significant value to increase the knowledge base. Close cooperation of researchers from different areas such as ecotoxicologists, environmental chemists, physicists, analytical chemists, material scientists, and also from industry can be highly beneficial (*e.g.* appendix 1; Handy 2008).

Most environmental data on ENPs available to date focus on ecotoxicology. However, it is still not clear exactly how, at which concentrations, and in what form ENPs will be released into the environment. It would be highly valuable to focus current research efforts on the release (*e.g.* fate of ENPs in sewage treatment), behaviour (*e.g.* reaction on change of environments such as change of pH, ionic strength etc.), and fate (*e.g.* mobility, aggregation, complexation, adsorption) of ENPs and base future ecotoxicological studies on these results.

Chapter 7

Overall conclusions and recommendations

Nanoparticles are a diverse class of new materials featuring extraordinary properties. The properties that make ENPs attractive in numerous applications might also present risks to humans and the environment. It has not been until recently, that ENPs have been identified as emerging environmental contaminants, thus research is sparse and more studies are needed.

In this PhD the importance of *in situ* ENP characterisation and analysis in environmental media to support environmental fate and ecotoxicity, and therefore ENP risk assessment has been shown. Analytical methods were identified to reliably detect and characterise nanoparticles and their properties in matrices to which humans and ecosystems are exposed, including air, soil and water as well as food and consumer products (chapter 2).

In chapters 3 and 4 novel analytical techniques were evaluated and developed to provide a better understanding of the environmental fate of nanoparticles:

It was found that WetSEM^{TM} has a great potential for *in situ* imaging of unperturbed environmental samples as seen by visualising particle-NOM interactions (chapter 3). Although the representativeness of WetSEM^{TM} images for the whole sample is unknown, the capsules allow qualitative observations on the major class of particles present in the sample; they can provide information on particle size and composition (in combination with EDS), state of aggregation and particle associations. The ability of visualising untreated, wet nanoparticle samples is a significant achievement and thus, WetSEM^{TM} can supply complementary information on the *in situ* investigation of particles in environmental matrices. However, the method is highly time

consuming and expensive and therefore could not be used in the fate investigations in this thesis.

As discussed in chapter 4, HDC-ICP-MS appears to offer a significantly improved approach for the characterisation of metal-based ENPs in complex environmental matrices, which was then confirmed by successfully applying the developed method to an ENP fate study. In comparison to other studies, where solely sub-micron microscopic techniques or total analysis were used, the application of HDC-ICP-MS provided additional information on the fate of nanosilver in sewage sludge showing NOM interactions or complexations of particles and dissolved particle species. For the first time, it could be shown that, although the majority of the silver partitioned to sewage sludge, a fraction of the silver remained in the supernatant in the nanoform and therefore could be released into the aquatic environment.

With the help of this data, it was then possible to predict the input of nanosilver from water treatment works into the environment, strengthening existing modelling approaches by producing real input data and providing essential information for ecotoxicologists and risk assessors on expected environmental concentrations of nanosilver in aquatic freshwater systems (chapter 5).

Based on the experience gained over the duration of this PhD project, current approaches for assessing and monitoring the impacts of ENPs on environmental systems were critically discussed. To increase the reproducibility of experimental data, it was found that an international testing program or guidance manual for engineered nanoparticles has to be urgently established including guidance on how to deal with different types and sources of nanomaterials (chapter 6).

While, the project has made significant developments in the area of risk assessment of engineered nanoparticles, there is an urgent need for future work.

Recommendations for future research building upon the work started in this PhD project include:

1. WetSEM^{TM} imaging

Due to resource constraints, the work described in the thesis has focused on imaging a few representative samples and images. A more detailed assessment of the methodology is required, in particular this should explore mechanisms for producing more quantitative information on a particular sample.

This study has focused on aqueous samples, yet the approach may have utility for other matrices such as soil, sediment, biota (*e.g.* invertebrate and fish tissue) and nanoparticle-containing products (*e.g.* cosmetics, food, paints). The study has also only looked at metal-based particles, its use for other particle types (*e.g.* carbon-based particles) should therefore be assessed.

Further investigations into particle-particle and particle-NOM interactions (*e.g.* properties that affect interactions) would be valuable to validate the observed interactions (Fe_2O_3 and TiO_2 nanoparticle interaction with NOM).

It may be possible to improve the resolution of the approach by optimising sample preparation or membrane coating and particularly imaging conditions (*e.g.* working distance, detector mode).

Although WetSEM^{TM} seems to be a promising tool, other sub-micron microscopic techniques should also be explored for their suitability to image nanoparticles in liquids. Cryo-TEM and WetSTEM are the most promising tools among these (chapter 2). Also a direct comparison of *e.g.* WetSEM^{TM} to ESEM would be valuable.

2. HDC-ICP-MS analysis

The HDC-ICP-MS approach has been developed to provide qualitative information on nanoparticles in aqueous samples. Further development of the approach is therefore required, so that the method can provide quantitative information on different size ranges. Quantitative data will be essential in the future in order to characterise the risks of engineered nanoparticles in aquatic systems.

The current size calibration approach has a number of limitations. It may be possible to improve the calibration by increasing the monodispersity of the size calibrants; also the size separation range of the column could be used to its full extent by synthesising stable and monodisperse Au nanoparticles up to 300 nm in size.

An improvement in the resolution of the column (type I; 5-300 nm) would be highly beneficial. This could be achieved by *e.g.* working closely together with the column manufacturer; this would be especially valuable, if the resolution could also be increased for column type II (20 – 1200 nm size separation range) as the poor resolution of column type II limits its application, however the wider size separation range would be advantageous.

In this study, the HDC-ICP-MS approach has been applied to aqueous samples. Further work is required to assess it application to analysis of other matrices such as foods, sediments, sludge solids and soils. In order to analyse nanoparticles in these other matrices, extraction methods will probably be required in order to get the sample into a form that is amenable to HDC-ICP-MS analysis. Such extraction methods will need to be extensively evaluated to ensure that the integrity of the particles of interest is maintained during sample processing.

By connecting the HDC column to other detector types (*e.g.* UV-Vis, MALLS), it may be possible to characterise other particle types (*e.g.* carbon-based). In addition, by combining these other detectors with ICP-MS detection, it may be possible to generate useful information on fate and behaviour processed *e.g.* NOM-particle interactions.

HDC-ICP-MS could be used alongside isotope labelling to differentiate ENPs from natural particles (*e.g.* radio labelling (*e.g.* ^{14}C), radio labelled tacking of inorganic nanoparticles, isotopic labelling (*e.g.* $^{64/67}Zn$)). This could be invaluable in

understanding the relative risks of natural and engineered particles in environmental systems.

Finally, the comparison of HDC-ICP-MS with complementary, but higher resolution methods such as flow field fractionation or size exclusion chromatography would be useful, *e.g.* the comparison of the Au calibrants. Also, techniques such as voltammetry should be explored and compared for their potential application to quantify dissolved nanoparticles.

3. Fate studies & modelling approaches to support environmental risk assessment

Environmental fate and ecotoxicological data are essential for the environmental risk assessment of ENPs. Further studies such as that presented in chapter 5 for a range of inorganic particles (coated and uncoated) and carbon-base particles are required. These studies should not only look at sludge but also fate in other systems. By applying several analytical techniques including methods such as HDC-ICP-MS (however, quantification by HDC-ICP-MS would be a major improvement), data on the concentration and characteristics of the particle of interest.

As discussed in chapter 6, the fate and ecotoxicity testing of engineered nanoparticles is highly challenging. Guidelines are therefore urgently needed for the experimental set-up as well as for particle characterisation. This will mean that data generated in different laboratories is more comparable that it is currently and hence that it is more useful for risk assessment purposes.

Alongside a lack of knowledge on the fate and ecotoxicity of ENPs, we have only a limited knowledge of what particles are used in different products and the concentrations (and form) of these particles in the products. Therefore, to support exposure modelling for ENP risk assessment, it would be valuable to develop more rigorous data bases on the production and use of engineered nanoparticles in different products. The development of emission scenarios for different product types would also be invaluable.

Despite all research efforts, the environmental behaviour, bioavailability, transport and transformation of ENPs is still widely unknown and, thereby, the potential impact on food webs and persistence (figure 7.1). Their effect on other substances also needs examination; for example, whether contaminant transport in the environment could be facilitated through adsorption to nanoparticles, whether nanoparticles enhance contaminant uptake or have a negative impact on bacteria useful for natural remediation.

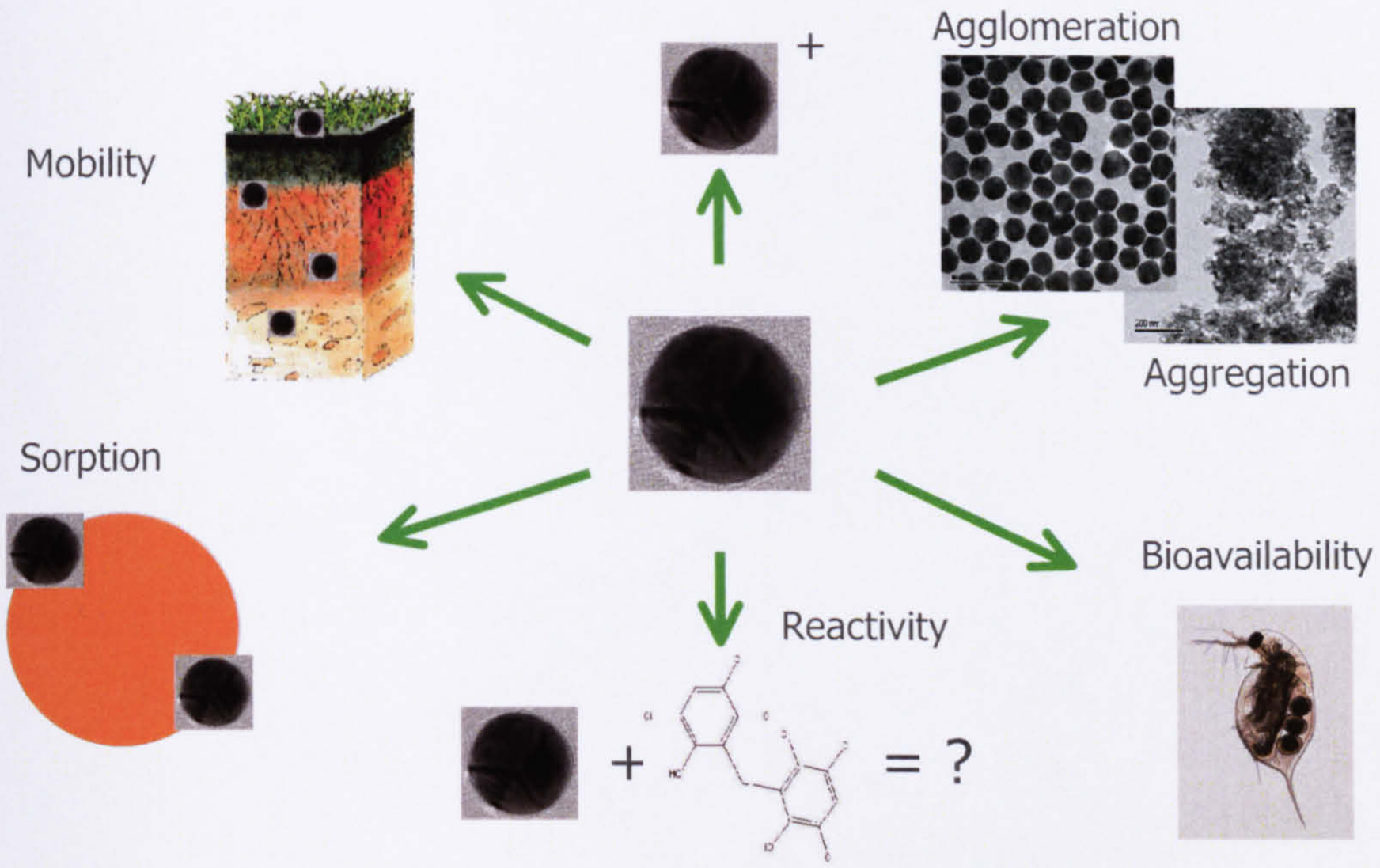


Figure 7.1. Environmental fate and behaviour of ENPs. The terms aggregation and agglomeration are used according to the definition by (Parfitt 1973).

Having said this, it has to be noted that the potential risks of nanotechnology have been recognised at an early stage. Knowledge gaps in environmental fate and (eco-) toxicity of ENPs have been identified and funding for research in this area is increasingly available. Thus, nanotechnologist, environmental scientists and ecotoxicologists, governments, industry and the public have the chance to find a balance to realize the benefits of nanotechnology and ensure human and environmental health amidst uncertain risks.

Appendices

Appendix 1

PERSPECTIVE

Engineered nanomaterials in soils and water: how do they behave and could they pose a risk to human health?

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It is inevitable that, during their use, engineered nanoparticles will be released into soils and waters. There is therefore increasing concern over the potential impacts of engineered nanoparticles in the environment on aquatic and terrestrial organisms and on human health. Once released into the environment, engineered nanoparticles will aggregate to some degree; they might also associate with suspended solids, sediment, be accumulated by organisms and enter drinking water sources and food materials. These fate processes are dependent on the characteristics of the particle and the characteristics of the environmental system. A range of ecotoxicological effects have also been reported, including effects on microbes, plants, invertebrates and fish. Although available data indicate that current risks of engineered nanoparticles in the environment to environmental and human health are probably low, our knowledge of the potential impacts of engineered nanoparticles in the environment on human health is still limited. There is therefore a need for continued work to develop an understanding of the exposure levels for engineered nanoparticles in environmental systems and to begin to explore the implications of these levels in terms of the ecosystem and human health. This will require research in a range of areas, including detection and characterization, environmental fate and transport, ecotoxicology and toxicology.

Nanotechnology is a rapidly expanding area and engineered nanomaterials/nanoparticles (ENPs) are finding applications in a wide range of areas, including use in cosmetics, bioremediation and water treatment (e.g., [1a,3]). It is therefore inevitable that, during their manufacture and use, ENPs will be released to the environment and they could then enter food and water supplies. Nanoparticles might also exist naturally (e.g., [4]), be formed in the water bodies (e.g., [5]) or be released to the environment in mine wastes (e.g., [6,7]). Concerns have therefore been raised over the potential impacts of indirect human exposure to ENPs on human health (e.g., [8,9]). In this article, we attempt to identify the major factors and processes that will affect the fate and effects of nanomaterials in the natural environment and identify the potential pathways of human exposure to ENPs in environmental matrices. Finally, we identify priorities for future research.

Inputs to the environment

Although ENPs might be emitted during the manufacturing process or by accidental spills, the route of input to the environment will depend primarily on the end use of the ENP (Table 1). For example, pharmaceuticals, cosmetics and sunscreens might be emitted to the sewage system following excretion from the patient or during washing and showering. Once they have passed through the sewer system, they might be released to surface waters. Sunscreens and other cosmetics applied to the skin might also enter surface waters directly during swimming or bathing. Waste cosmetics are most likely to be disposed of in household waste that might be landfilled or incinerated. Paints containing ENPs can have both industrial and domestic uses. It is possible that run-off from painted surfaces and domestic use of paints could result in discharges to sewers. In instances in which paint is applied to underwater structures or ships, ENPs might be released directly to surface waters. The use of ENPs in fuel and catalysts in vehicles will result in direct aerial emission of particles through vehicle exhaust or emissions to the surface waters by aerial deposition, leakage and spills. Waste lubricants are most likely to be disposed of as special waste that might be landfilled or incinerated. The use of nanoparticles in the treatment of polluted water is likely to result in direct emissions to surface and groundwaters or soil. ENPs used to deliver agrochemicals will be released directly to soils and surface waters.

Fate & behavior of ENPs in the environment

Over the past few years, there has been increasing interest in the environmental behavior of

Keywords: ecotoxicity, engineered nanoparticles, environmental exposure, environmental fate, risk, soil, water

future Medicine part of fsg

Table 1. Major routes of input of engineered nanomaterials to the environment for different sectors and applications.

Sector/application	Nanomaterial type	Probable exposure routes					
		Air	*Surface water*	*Ground water*	*Wastewater*	*Soil*	*Waste*
Cosmetics and personal-care products	TiO_2, ZnO, fullerene (C_{60}), Fe_2O_3, Ag		√		√		
Catalysts, lubricants and fuel additives	CeO_2, Pt, MoS_3	√	√		√		
Paints and coatings	TiO_2, SiO_2, Ag, quantum dots	√	√		√		
Water treatment and environmental remediation	Fe, Fe-Pd, polyurethane		√	√	√	√	
Agrochemicals	SiO_2 (porous) as a carrier	√	√			√	
Food packaging	Ag, nanoclay, TiO_2					√	√
Pharmaceuticals and medicines	Nanomedicines and carriers			√	√	√	

ENPs in water and soils. Once a nanoparticle is introduced into the water environment, there are many processes that might affect their fate, including partitioning to sediment and suspended particulate material, biological degradation (aerobic and anaerobic) and abiotic degradation (including photolysis and hydrolysis). A number of studies have investigated the fate and transport of engineered nanoparticles in water systems. Most of this work has focused on the aggregation behavior of the ENPs and indicates that, following release to water, nanoparticles (including carbon nanotubes, nanoscale zerovalent iron, titanium dioxide and fullerenes) will aggregate to some degree (e.g., [10,13]) (Table 2). The degree of aggregation and the size range of the aggregates is dependent on the characteristics of the particle (i.e., type, size and surface properties) and the characteristics of the environmental system (including pH, ionic strength and dissolved organic carbon content) [11,12,14]. Aggregation reduces the specific surface area of the particles and the interfacial free energy, and will therefore reduce particle reactivity [15]. A number of modeling approaches have been proposed for predicting aggregation behavior in aquatic systems [16]; however, these have yet to be fully evaluated. It is also important to recognize that stabilizers might be used in the manufacturing process to reduce agglomeration and enhance the dispersion of engineered nanoparticles within a product. The stabilizers work by either electrostatic repulsion, in which a charged stabilizer is adsorbed to the particle increasing repulsion between particles; or steric hindrance, in which a bulky stabilizer is used to impede particle attraction. A wide range of stabilizers is effective, including thiols, carboxylic acids, surfactants and polymers. The stabilizers not only affect the behavior of the engineered nanoparticle within a product but are also likely to affect behavior in the environment (e.g., [17]).

Information on the interaction of free and aggregated engineered nanoparticles with suspended solids and sediments is lacking from the literature, although some studies suggest that, following aggregation, selected ENPs might sediment out and hence it is likely that they will associate with bed sediments [13]. Information on the persistence of ENPs in environmental systems is also not available readily, although some polymer-based particles are known to be degradable in other biological systems and therefore might be expected to be degraded in the natural environment [18,19].

Following release to the soil environment, ENPs might sorb to soil particles, be degraded by biotic and abiotic processes and be transported to water bodies through runoff, leaching and drainflow. Some experimental data are available on the sorption and transport behavior of ENPs in soils. For example, sorption studies with selected ampiphilic polyurethane nanoparticles have demonstrated that these particles strongly sorb to soil particles [17]. Information is available on the transport of nanotubes, fullerols, fullerenes and polyurethane ENPs. Data from simple laboratory studies indicate that mobility varies depending on nanomaterial type [20]; for example, single-walled nanotubes (SWNTs) and fullerols appear to pass through porous media more quickly than

Table 2. Size distribution of selected engineered nanoparticles in water.

Nanopartide type	Mean partide size (nm) (range is given in parentheses)	Ref.
C_{60}	75 (25–500)	[10,41]
TiO_2	330 (175–810)	[42]
SiO_2	205 (135–510)	[42]
ZnO	480 (420–640)	[42]
Zerovalent Fe	>1000	[15,43–44]
Fullerol	100	[13]

C_{60}. Other studies with ampiphilic polyurethane nanoparticles showed these particles to be mobile through a sandy aquifer material. Environmental conditions, such as pH of the surrounding environment, might also be very important in determining the degree of transport (e.g., [11]). The fate of the mobility of the materials might also be affected by the environmental modifications, such as capping and wrapping of carbon nanotubes with surfactants or the functionalization of C_{60} [20].

Although the data on the fate of ENPs in the environment are still limited, the behavior of colloidal material, which will be in the nanoscale size, in the soil environment has been studied extensively and models have been developed for predicting behavior (e.g., [11,21]). Transport in porous media can be described by three mechanisms: interception of the particle by the media, sedimentation owing to gravity and diffusion from Brownian motion, and deposition. It is possible that these approaches could be applied to predict the behavior of ENPs in the soil environment. However, the extent to which engineered nanoparticles behave differently to colloids has yet to be established.

Effects of ENPs in the environment

Alongside the fate investigations, studies have explored the uptake and effects of nanoparticles on a range of environmental species and end points [22ñ25], including invertebrates, fish, algae and bacteria (Table 3). In the laboratory, aquatic organisms, such as invertebrates, appear to accumulate selected engineered nanoparticles rapidly, including carbon black, titanium dioxide and polystyrene (e.g., [26,27]).

When acute end points (i.e., mortality) are considered, median effect concentrations for those ENPs studied to date, including fullerenes, titanium dioxide and SWNTs, are generally in the mg/l range (Table 3). A range of sublethal effects have also been studied, including effects on invertebrate heart-rate appendage movement, growth and reproduction; effects on fish-protein expression, hematology and metal-ion concentrations; and effects on bacterial growth and phospholipid fatty-acid profile (e.g., [10,28]). For the ENPs studied so far, these sublethal effects are observed typically in the µg/l range.

In some cases, there is a mismatch between results of laboratory studies and studies to assess impacts in the real environment. For example, under realistic exposure conditions, fullerenes have little impact on the structure and function of the soil microbial communities and microbial processes [29], whereas effects are observed in simple laboratory investigations. These differences are probably explained by changes in bioavailability and the form of the engineered nanoparticles under more natural conditions.

Just like exposure, the factors and processes affecting ecotoxicity seem to be complex. The impacts of ENPs on environmental organisms seem to be determined by a range of characteristics, including dissolution potential, aggregation potential, particle surface properties and the characteristics of the exposure environment and the biochemical, physiological and behavioral traits of the organism of interest (e.g., [30,31]).

Could ENPs pose a risk to organisms in the environment?

Recent studies in our laboratory [32] have attempted to assess the potential environmental concentrations for a range of current ENP types. Using product characteristics and usage information, potential concentrations of C_{60}, Ag and AlO_3 ENPs in water were estimated to be in the ng/l range, whereas predictions of potential TiO_2, SiO_2 and ZnO ENP concentrations in water were in the low µg/l range. A comparison of these predictions with the available data on toxicity to aquatic organisms indicates that concentrations of existing ENPs in the environment are likely to be significantly lower than concentrations required to cause both lethal and sublethal effects, and that the risk to the soil and aquatic environments from current applications of ENPs is low. The fact that many of the ENPs will agglomerate in environmental systems and that many of the ecotoxicological studies have been performed to optimise toxicity (e.g., by attempting to maintain test ENPs as close to the free form as possible) provides a further margin of safety.

Table 3. Available ecotoxicity data for a range of engineered nanopartides .

ENP	Test species	End point	Result (mg/l)	Ref.
Fullerene C60	Daphnia magna	48 h LC50 (mortality)	>35 for water-stirred C_{60} 0.8 for THF-applied C_{60}	[45]
	Daphnia magna	Hopping, heart rate appendage movement	Effects observed at 0.26 mg/l	[46]
	Daphnia magna	21 day mortality, reproduction and molting	40% mortality observed at 35 mg/l; effects on molting and reproduction observed at 2.5 mg/l	[24]
	Hyallela azteca	96 h mortality	No effect at 7 mg/l	[24]
	Copepods	96 h mortality	No effect at 22.5 mg/l	[24]
	Pimephales promelas	96 h mortality and sublethal effects	No mortality at 0.5 mg/l; PMP70 protein expression suppressed at 0.5 mg/l but no effect on CYP1A, 2M1 and 2K1 levels	[24]
	Oryzias latipes	96 h mortality and sublethal effects	No mortality at 0.5 mg/l; no effect on CYP1A, 2M1 and 2K1 PMP70 protein levels at 0.5 mg/l	[24]
	Escherichia coli	Growth	No growth at 0.4 mg/l; growth at 0.04 mg/l	[10]
	Escherichia coli	Respiration	Inhibition at 4 mg/l; no inhibition at 0.4 mg/l	[10]
	Bacillus subtilis	Growth	No growth at 0.4 mg/l; growth at 0.04 mg/l	[10]
	Bacillus subtilis	Respiration	Inhibition at 4 mg/l; no inhibition at 0.4 mg/l	[10]
	Bacillus subtilis	Phospholipids and membrane-phase behavior	Effects observed at 0.01 mg/l	[28]
	Bacillus subtilis	Minimal inhibitory concentration	nC_{60} = 0.4–0.6 mg/l; 'small' nC60 particles = 0.1–0.23; 'large' nC60 particles = 0.75–1.5	[41]
	Pseudomonas putida	Phospholipids and membrane-phase behavior	Effects observed at 0.01 mg/l	[28]
	Soil microbes	Community structure and function	Little effect at 1 mg/kg (nC_{60}) and 1000 mg/kg (C_{60})	[29]
TiO_2	Oncorhyncus mykiss	96 h LC50 (mortality)	>100	[47]
	Daphnia magna	48 h EC50 (immobilisation)	>100	[47]
	Pseudokirchneriella subsapitata	72 h EC50 (growth)	16	[47]
	Daphnia magna	Hopping, heart-rate appendage movement	No effects observed at 2.0 mg/l	[46]
	Bacillus subtilis	Growth inhibition	No inhibition at 500 mg/l; 75% inhibition at 1000 mg/l	[42]
	Escherichia coli	Growth inhibition	No inhibition at 100 mg/l; 15% inhibition at 500 mg/l	[42]
SWNTs	Amphiascus tenuiremis	28–35 day EC_{50} (mortality, development, reproduction)	>10 (effects on mortality, fertilization and molting rates observed at 10 mg/l)	[48]
	Oncorhyncus mykiss	10 day sublethal effects	Effect on respiration at 0.1 mg/l; no major disturbance to hematology; changes in brain and gill Zn and Cu; increase in Na^+K^+-ATPase	[49]

ENP: Engineered nanoparticle; LPC-SWNT: Lipid-coated-single-walled nanotube; SWNT: Single-walled nanotube.

Table 3. Available ecotoxicity data for a range of engineered nanoparticles (cont.).

ENP	Test species	End point	Result (mg/l)	Ref.
LPC-SWNTs	Daphnia magna	96 h mortality	100% mortality at 20 mg/l; 20% at 10 mg/l; no mortality at 5 mg/l	[50]
$C_{60}HxC_{70}Hx$	Daphnia magna	Hopping, heart-rate appendage movement	Effects observed at 0.26 mg/l	[46]
SiO_2	Bacillus subtilis	Growth inhibition	No inhibition at 500 mg/l; 7% inhibition at 1000 mg/l	[42]
	Escherichia coli	Growth inhibition	No inhibition at 100 mg/l; 15% inhibition at 500 mg/l	[42]
ZnO	Bacillus subtilis	Growth inhibition	90% inhibition at 10 mg/l	[42]
	Escherichia coli	Growth inhibition	14% inhibition at 10 mg/l	[42]

ENP: Engineered nanopartide; LPC-SWNT: Lipid-coated-single-walled nanotube; SWNT: Single-walled nanotube.

Could ENPs in soils & waters pose a risk to human health?

Although we are beginning to understand the potential impacts on aquatic and terrestrial organisms, little consideration has been given to the potential human-health risks of the exposure to ENPs in the environment. Based on our understanding of other chemicals in the environment, possible major routes of exposure will include the consumption of contaminated soil, water or the consumption of food products (Figure 1) grown in environments contaminated with ENPs. For nanoparticles released to the sewage system (e.g., ENPs used in personal-care products or as pharmaceuticals), the potential for transport of ENPs into aquatic systems has yet to be demonstrated. The limited data available for soils indicate that ENPs applied to soils (e.g., pesticides or veterinary medicines) could be transported into water bodies (e.g., [20]). Once in water sources, they might have the potential to pass through typical drinking-water treatment processes. Although selected aquatic organisms also appear to accumulate selected nanoparticles rapidly, including carbon black, titanium dioxide and polystyrene (e.g., [26,27]), uptake into major food items (e.g., plants) has not yet been studied. Moreover, the potential transfer through food chains has yet to be established.

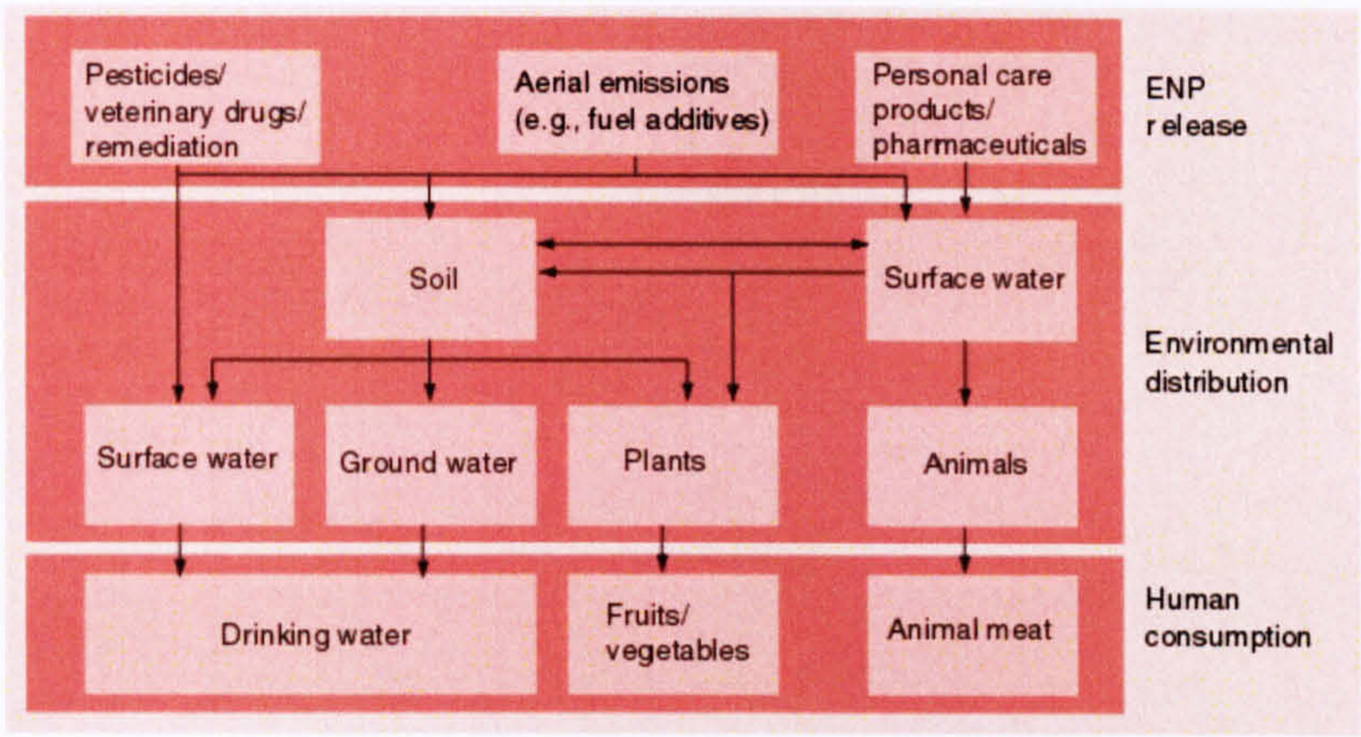
As discussed earlier, concentrations in water and soil arising from existing applications of ENPs are likely to be low. Therefore, for many ENPs, the exposure through the soil environment might be insignificant compared with exposure arising through product use (e.g., personal-care product use, paint application and drug administration), although it is important to note that the route of entry into the body will be different (i.e., uptake through the skin vs oral intake). Moreover, because many ENPs also aggregate in the environment, any toxic properties that are associated with the particle size might also be nullified.

It is important, however, to recognize that, as more applications for existing ENPs are found and as new ENPs come onto the market, the risks to humans and the environment could increase. There is therefore the need for continued work to establish what the potential environmental exposure is now and in the future, major exposure routes for humans, if any; and the relative risks of environmental exposure to humans compared with other exposure routes. We would advocate that this work should focus on the following areas.

Detection

Properties, such as the dynamics of dispersion, rate of dissolution, characteristics of the nanoparticle aggregates, surface area and surface characteristics are all likely to affect the behavior and effects of engineered nanoparticles in environmental systems [33]. Therefore, when assessing environmental exposure, it will not only be necessary to measure nanoparticle concentrations but it will also be necessary to develop an understanding of the morphology, size distribution and surface properties. Several techniques have been applied for the physical and chemical characterization of nanoparticles. Detection and characterization methods have included infrared (IR), ultraviolet (UV), fluorescence, mass spectrometry (MS), inductively coupled plasma mass spectroscopy (ICP-MS), nuclear magnetic resonance spectrometry (NMR), x-ray spectroscopy and dynamic-light scattering, as well as imaging techniques, such as scanning-electron microscopy (SEM), wet-SEM, transmission-electron microscopy (TEM) and atomic-force

Figure 1. Potential indirect exposure routes for different applications of engineered nanoparticles.



ENP: Engineered nanopartide.

microscopy (AFM) (e.g., [34,35]). Many of these detection techniques have been combined with separation methods, such as flow field fractionation (FFF), high-performance liquid chromatography, gel-permeation chromatography and size-exclusion chromatography (e.g., [34,36]). There is now an urgent need to further develop these approaches for environmental systems.

Fate & transport in environmental systems

The available data indicate that the behavior and transport of ENPs will be affected by the properties of the ENP and the characteristics of the surrounding environment. Research is required to develop a mechanistic understanding of the fate of ENPs in water, soil, sediment and wastewater in order to provide information on interactions with themselves, other ENPs, suspended solids, dissolved organic material and on how these interactions are affected by environmental variables (including pH and ionic strength). The potential for ENPs to act as carriers for other environmental contaminants, such as pesticides and persistent organic pollutants, should also be established.

Environmental exposure assessment

We need to establish urgently the current and potential future level of ENPs in environmental media and the potential human exposure resulting from these levels. This will require the development of models and model scenarios to assess not only exposure concentrations, but also the characteristics of ENPs once they are released to the natural environment. Guidance already exists for assessing the environmental exposure for substances in different sectors (e.g., [37ñ39]). This existing guidance makes little reference to the assessment of substances in the particulate form; however, it is possible that some of the more simple models that exist could be used to provide a ëworst caseí estimate of environmental exposure. This will give us a benchmark against which we can compare ecotoxicity and toxicity data. As our knowledge of fate and behavior increases, it should be possible to develop more complex modeling approaches.

Uptake of ENPs into food items

Laboratory studies demonstrate that selected ENPs might be taken up by organisms in the environment. We now need to develop a thorough understanding of potential uptake from water into fish and from soils into plant material, as well as the potential for trophic transfer of nanomaterials. This information can be used to evaluate the applicability of existing modeling approaches for secondary poisoning and will provide valuable information on the relative importance of indirect environmental exposure in terms of human health. The bioaccessibility, and factors affecting this of the ENPs from these different food materials should also be established.

Many of these research needs are relevant to the ëgrand challengesí identified by Maynard *et al.* [40]. These studies will be challenging and will require the input from ecotoxicologists, exposure modelers, analytical chemists, physicists, food chemists, toxicologists and those involved in the development of new ENP applications.

Future perspective

Nanotechnology is a rapidly developing field so the level of risk could change in the future as new applications are found and new ENPs are introduced. There is therefore still a need to better understand the fate and transport, exposure and effects of engineered nanoparticles in the environment and to integrate these different areas to determine the risk to ecosystem and environmental health.

Work in the near future should focus on:

- The detection and characterization of engineered nanoparticles in soil and water;
- Developing a detailed understanding of environmental fate and transport processes;
- The development of approaches for assessing human and ecosystem exposure to engineered nanoparticles;
- The combination of this information with ecotoxicological and toxicology data to establish risks to ecosystem and human health.

Executive summary

- It is inevitable that, during their use, engineered nanoparticles will be emitted to aquatic and terrestrial systems.
- The characteristics of engineered nanoparticles in the environment might be very different from in the original products – they might aggregate, sorb to solid material, associate with other environmental contaminants or be accumulated. This behavior will be affected by the particle type and size, as well as the nature of the environmental system of interest.
- Ecotoxicological effects have been reported on a range of aquatic and soil organisms. Levels of environmental exposure, arising from existing applications of ENPs, are, however, likely to be much lower than those required to cause ecotoxicological effects.
- We know little currently about the risks to human health of exposure to ENPs in water and soil. Based on our existing knowledge, it is possible that environmental exposure will be insignificant compared with other exposure routes (e.g., product use) and that aggregation in the environment will remove or reduce any special toxicological properties of a particle.

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Appendix 2

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Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles

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Abstract Environmental risk assessments of engineered nanoparticles require thorough characterization of nanoparticles and their aggregates. Furthermore, quantitative analytical methods are required to determine environmental concentrations and enable both effect and exposure assessments. Many methods still need optimization and development, especially for new types of nanoparticles in water, but extensive experience can be gained from the fields of environmental chemistry of natural nanomaterials and from fundamental colloid chemistry. This review briefly describes most methods that are being exploited in nanoecotoxicology for analysis and characterization of nanomaterials. Methodological aspects are discussed in relation to the fields of nanometrology, particle size analysis and analytical chemistry. Differences in both the type of size measures (length, radius, aspect ratio, etc.), and the type of average or distributions afforded by the specific measures are compared. The strengths of single particle methods, such as electron microscopy and atomic force microscopy, with respect to imaging, shape determinations and application to particle process studies are discussed, together with their limitations in terms of counting statistics and sample preparation. Methods based on the measurement of particle populations are discussed in terms of their quantitative analyses, but the necessity of knowing their limitations in size range and concentration range is also considered. The advantage of combining complementary methods is highlighted.

Keywords Nanoparticles · Nanoaggregates · Nanometrology · Analytical chemistry · Particle size analysis

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Introduction

Due to the extensive current, and foreseen future investments, in nanotechnology, nanoparticles used in consumer products, industrial applications and health care technology are likely to enter the environment (Aitken et al. 2006; Roco 2005). To ensure sustainable development of nanotechnology, there is a need for risk assessments of engineered nanoparticles (ENP) introduced from various applications (Colvin 2003; Maynard et al. 2006). Such risk assessments, require proper tools and methodologies to carry out both effect and exposure assessments (EPA 2007; Maynard et al. 2006; SCENIHR 2005; Crane and Handy 2007). Conventionally, exposure assessment is recommended to include both a modeling and a measurement approach (Holt et al. 2000); both approaches require instrumentation and analytical methods. Prediction of environmental concentrations of ENP through modeling is based on emission scenarios (from production volumes and

life cycle assessments) and partitioning parameters (fate and behavior). Presently, little is known about the fate and behavior parameters of ENP. Hence, development of suitable analytical methods are required to determine concentrations and nanoparticle characteristics in complex environmental matrices such as water, soil, sediment, sewage sludge and biological specimens. The approach for prediction of environmental concentrations through modeling requires validation through measurement of actual environmental concentrations. For ENPs that are only recently being introduced into the environment, extremely sensitive methods are required. Although direct observations are not hampered by the underlying assumptions of exposure modeling, it is very important to assure that direct observations are representative in time and space for the regional setting to which the observation will be allocated (local or regional).

ENP differ from most conventional "dissolved" chemicals in terms of their heterogeneous distributions in size, shape, surface charge, composition, degree of dispersion, etc. Therefore, it is not only important to determine their concentrations, but also several other metrics.

In addition to exposure assessment requirements, it is essential that characterization of ENP dispersion states (i.e. aggregated or dispersed), and measurements of "steady-state" concentrations are used in effect assessment test systems (e.g., toxicity testing). It has been found that the ENP concentrations are often not sustained in dispersions throughout an experiment (Federici et al. 2007). Although this need was not recognized in the pioneer studies in nanoecotoxicology, it is now starting to be implemented in most effects experiments. In a recent review (Hansen et al. 2007), it was shown that although size determinations are becoming more common (17–96% of exposure and effects studies), other relevant characterization properties are rarely determined (e.g., surface area in only 6–33% of studies). An additional complication relates to stability. For example, Fig. 1 demonstrates that Buckminster fullerenes readily degrade and are highly reactive (Taylor et al. 1991). Indeed, it is the reactions of Buckminster fullerenes that render them of particular interest when investigating their potential applications in nanotechnology (Taylor 2006). This reactivity has substantial implications in interpretation of environmental behaviour and ecotoxicological impact.

Assessing uptake and bioaccumulation in biological matrices are essential and will be equally as challenging as analyses of complex environmental media. Furthermore, some good laboratory practices and harmonized methods still need to be developed. Due to both the complexity of the behavior of nanomaterials in dispersions and the requirements for expertise in state-of-the-art methods in ecotoxicology testing and nanoparticle characterization, the necessity for interdisciplinary collaboration has been highlighted (Crane and Handy 2007; Handy et al. 2008). This paper focuses on mature and validated methods that are commercially available and/or fairly easy to setup. Consequently, highly specialized methods in the development phase, or methods requiring large-scale facilities such as synchrotron sources, are not discussed.

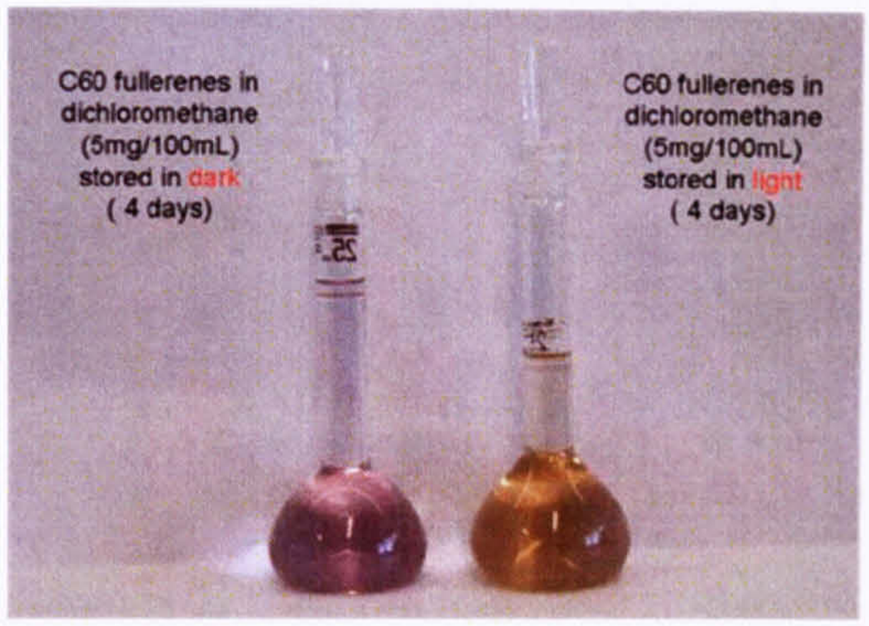

Fig. 1 C60 fullerene solutions (in toluene) stored under dark and light conditions (Photograph courtesy of P. Frickers and J.W. Readman). The photo illustrates the potential of using spectroscopic methods to study these photochemical changes in structure or surface chemistry

Nanometrology, analytical chemistry and particle size analysis

The physical properties of nanoparticles are referred to as metrics (Table 1) and the field of science which aims to standardize physical measurements at the nanometer scale, is called nanometrology. Even though nanometrology is a young field regarding definitions and terminology, many concepts are borrowed and adopted from the fields of particle size analysis (Barth and Flippen 1995) and physical chemistry. In addition to the physical properties, nanoparticles can be described by their chemical composition where the compound or species determined is called the analyte (Table 1).

The metric "particle diameter" is probably the most commonly used descriptor of particle size, but a single diameter value is only enough to describe a perfect spherical particle. Non-spherical nanoparticles (or colloids) are, however, common in the environment and it is actually common for nanoparticles to have very large aspect ratios (e.g., clay platelets, rods or fibrils). Many engineered nanoparticles share these features (e.g., carbon nanotubes, nanowires, nanoclays, nanorods). It has been shown that the toxicity can be shape dependent (Pal et al. 2007), and

Table 1 A list of physical properties (metrics), and a list chemical compositions, analytes and respective associated methods and instruments

	Instruments and methods[a]
Physical properties/metrics	
Diameter	EM, AFM, Flow-FFF, DLS,
Volume	Sed-FFF
Area	EM, AFM
Mass	LC-ESMS
Surface charge	z-Potential, electrophoretic mobility
Crystal structure	XRD, TEM-XRD (SAED)
Aspect ratio or other shape factor	
Chemical composition/analytes	
Elemental composition	Bulk: ICP-MS, ICP-OES, single nanoparticle: TEM-EDX, particle population: FFF-ICP-MS
Fluorophores	Fluorescence spectroscopy
Fullerene ("molecules")	UV–vis, IR, NMR, MS, HPLC
Total organic carbon	High temp chemical oxidation
Other properties not falling within the above classes	
Aggregation state	DLS, AFM, ESEM, etc.
Hydrophobicity	Liquid–liquid extraction chromatography
Dissolution rate	Dialysis or voltammetry or spectrometry
Surface chemistry, coating composition, # of proton exchanging surface sites	Optical or X-ray spectroscopic methods, acid–base titrations

[a] For abbreviations see text

nanoparticle reactivity can be dependent both on size and shape (Madden and Hochella 2005). There are several different diameter measures that correspond to an equivalent size of a specific type (Table 2). Different particle size analysis methods also yield different equivalent sizes (Table 2), which is important to consider when comparing size values obtained using different methods. Another important feature in method comparisons is that different techniques give different size averages, depending on if they fundamentally rely on an instrument response to: particle numbers, volume, mass or optical property (e.g., light scattering) (Table 3). These averages can be the same for spherical, monodisperse particles (with an infinitely narrow size distribution) This, however, is usually not the

Table 2 Different equivalent sizes measured by different methods

Equivalent spherical size measures	Applies to method	
Hydrodynamic diameter	Flow-FFF, DLS	Calculated from the measured diffusion coefficient, using Stokes–Einstein equation
Equivalent spherical volume diameter	Sed-FFF (if known density), LIBD, electrozone sensing	
Buoyant mass	Sed-FFF	SedFFF $\propto \Delta\delta^{*}V$
Equivalent spherical mass diameter	MS	Assume a certain structure
Projected area	Microscopy	
Equivalent molar mass	Ultrafiltration	Molecular weight cutoff (MWCO), defined from retention of proteins
Equivalent poresize diameter	Particle filtration	Filter poresize often defined as maximum size that penetrates filter
Root mean square radius of gyration	SLS	mean square distances from center of mass of point masses within the particle
Aspect ratio	Microscopy, combination of light scattering methods or different FFF methods	The longest dimension divided by the shortest for symmetrical particles (e.g., rods & ellipsoids)

Table 3 Description of different types of size averages, with equations defining them and methods that are deriving such average sizes

Type of size average	Applies to method	Equation
Number average: size average of numbers of particles within a certain size class	Microscopy, LIBD	$\bar{d}_n = \frac{\sum n_i \cdot d_i}{\sum_i n_i}$
Mass or volume average: size average of volume of particles within a certain size class	FFF and SEC with most detection methods, CFF	$\bar{d}_v = \frac{\sum V_i \cdot d_i}{\sum_i V_i}$
Z-average size, an intensity weighted average attributed to certain methods	Dynamic light scattering	$\bar{d}_z = \frac{\sum n_i \cdot d_i^6}{\sum n_i \cdot d_i^5}$

case. Each method also has its limitations in applicable size and concentration ranges (Table 4). Therefore, it has to be taken into account that there may be part of the nanoparticle (or nanoparticle-aggregate) size distribution that is "hidden" for the applied method. Some relevant terms and definitions from analytic chemistry, nanometrology and particle size analysis is given in Table 5.

There are some special challenges for studies of ENPs in environmental samples. The first challenge is that for environmentally relevant concentrations (ng l^{-1}–pg l^{-1}), the detection limits for most methods are not sufficiently low. The second challenge is that in environmental samples there is a high background of natural and unintentionally produced nanoparticles (Banfield and Navrotsky 2001; Filella 2007; Hochella and Madden 2005; Lead and Wilkinson 2006; Waychunas et al. 2005; Wigginton et al. 2007).

A strategy for coping with these challenges may be to combine existing and new methods that afford both a screening capability and a highly selective detection. These techniques, however, can be developed and tested under less stringent experimental conditions (with higher concentrations) to investigate behaviors, fates and effects.

Table 4 Specifications of methods for analysis and characterization of nanoparticles

Method	Approximate size range (nm)	Limit of detection[a]	Single particle or particle population methods	Level of sample perturbation
AFM	0.5 to >1000	ppb–ppm	sp	Medium
BET	1 to >1000	Dry powder	pp	High
Centrifugation	10 to >1000	Detection dependant	pp	Low
Dialysis	0.5–100	Detection dependant	pp	Low
DLS	3 to >1000	ppm	pp	Minimum
Electrophoresis	3 to >1000	ppm	pp	Minimum
EM-EELS/-EDX	Analysis spot size: ~1 nm	ppm in single particle	sp	High
ESEM	40 to >1000	ppb–ppm	sp	Medium
ES-MS	<3	ppb	pp	Medium
FFF	Flow FFF: 1–1000 Sed FFF: 50–1000	Detection dependant; UV: ppm, Fluo&ICP-MS: ppb	pp	Low
HDC	5–1200	Detection dependant	pp	Low
ICP-MS	Depends on fractionation	ppt–ppb	pp	
LIBD	5 to >1000	ppt	sp	Minimum
Microfiltration	100 to >1000	Detection dependant	pp	Low-medium
SEC	0.5–10	Detection dependant	pp	Medium
SEM	10 to >1000	ppb–ppm	sp	High
SLS	50 to >1000		pp	Minimum
TEM/HR-TEM	1 to >1000	ppb–ppm	sp	High
TEM-SAED	Analysis spot size: 1 nm		sp	High
Spectrometry		ppb–ppm	pp	Minimum
Turbidimetry/nephelometry	50 to >1000	ppb–ppm	pp	Minimum
Ultrafiltration	1–30	Detection dependant	pp	Medium
WetSEM	50 to >1000	ppm	sp	Low
WetSTEM		ppm	sp	Low
XRD	0.5 to >1000	Dry powder	pp	High

[a] For comparison mass concentration limit of detection for 100 nm particles are estimated

Table 5 Analytical chemistry, metrology and particle size analysis definitions

Term	Definition
Metric	The property that is being quantified
Analyte	The compound or specie that is being quantified
Limit of detection	The lowest concentration that can be distinguished from the background, typ defined as 3*Stdev (blank measurements)
Precision	The statistical spread of values in a measurement series
Accuracy	The exactness of the averaged measurements related to the true value
Measurement uncertainty	The accumulated uncertainty incl. method, lab, between days and between lab biases
Method validation	Experimental proof that the method conforms according to the specifications
Reference material	A material or substance that is sufficiently homogeneous for its property values to be used for calibration of instruments or assessment of methods
Certified reference material	A reference material that is accompanied by a certificate that specifies the traceability of the CRM and associated uncertainty
Control sample	Within laboratory quality control over time and between interlaboratory comparisons
Interlaboratory comparison	A blind test between participating laboratories to quantify deviation from true or reference value
Number based concentration	Determinations of number of particles per unit volume or mass
Mass based concentration	Determinations of mass of particles per unit volume or mass
Number average based size	The size average of numbers of particles within a certain size class: $\bar{d}_n = \frac{\sum n_i * d_i}{\sum_i n_i}$
Volume average based size	The size average of volume of particles within a certain size class: $\bar{d}_v = \frac{\sum V_i * d_i}{\sum_i V_i}$
Z-average based size	A light scattering based average: $\bar{d}_n = \frac{\sum n_i * d_i^6}{\sum_i n_i * d_i^5}$
Polydispersity index	Weight average size/number average size

Dispersion, sampling and sample handling

Dispersion of nanoparticles for both exposure and effect assessments

Colloidal systems are dynamic non-equilibrium systems and are often sensitive to physical or chemical disturbances (Filella 2007). Sampling and laboratory procedures (e.g., pumping, mixing, etc.) that introduce shear forces are likely to perturb the dispersion state of ENPs, possibly leading to either further aggregation, or to partial disruption of existing aggregation. The presence of natural organic matter and natural nanoparticles further complicates the situation. It is important to be aware of and characterize the interaction of the ENP with the natural material. It is equally important to compensate for any background material of the same composition as the ENP. Background levels of identical composition can be present for TiO_2, SiO_2 but also for carbon-based nanoparticles. Geological studies, using primarily transmission electron microscopy (TEM) to visualise the materials, have reported fullerenes in geological formations dating back 1.85 billion years (Becker et al. 1994), and CNTs together with fullerene–like structures in a Greenland ice core dated at approximately 10,000 years old (Murr et al. 2004). Given their reactivity, this is surprising (Taylor 2006), but infers that these carbon-based nanoparticles have natural as well as engineered origins.

In the case of ecotoxicological exposures to carbon nanoparticles, the preparation and characterisation of aqueous fullerene suspensions is especially challenging owing to their low solubilities. Fortner et al. (2005) describe nano-aggregate formation of C60 fullerenes in water. Particle sizes within the aggregates are, however, dependant on formation parameters including pH, ionic strength and even the mixing rates. The properties of the aggregates are different from the pristine particles. Coupled with the fact that fullerenes oxidise (Fig. 1), ecotoxicological exposure techniques are rendered highly complex. For carbon nanotubes (CNTs), their extremely low solubility in water, variable sizes of the particles, small diameters and the complexity of aggregates formed render dosing and particulate characterisations extremely difficult in aqueous exposure experiments. Nowack and Bucheli (2007) describe a standard procedure for solubilising CNT through cutting the tubes by sonication, and hydroxylation of the ends and damaged regions using strong acid. Other treatments to disperse the materials are reported using surfactants (Jiang et al. 2003) and biopolymers, including humic and fulvic acids (Hyung et al. 2007).

Treatments to facilitate dispersion must, however, be accounted for in interpretation of toxic response and how environmental relevance may be affected.

Sampling

Due to the unstable nature of colloidal nanoparticle dispersions it is preferable to use in situ analyses, but these methods are rarely available (Lead and Wilkinson 2006). The second choice is to apply methodologies that cause minimum perturbation from sampling to analysis. An example of such techniques are the probing of dispersions with electromagnetic radiation (e.g., light, X-rays or neutrons) where the scattering/absorption patterns can be related to physical properties of the particles, as will be described below.

Sample contamination and loss

Sampling of nanoparticles should generally be feasible with most standard sampling protocols, but the handling procedures differ from many other chemicals. Samples of colloids from surface waters are often collected in bottles that have been selected for minimum adsorption and contamination, e.g., plastics, especially fluoroplastics, for inorganic colloids or metal analysis and glass for analysis of organic trace constituents (Hall 1998). Since engineered nanoparticles may consist of e.g., an inorganic core with an organic coating or surfactants, conventional material selections may have to be revised. Further, the nanoparticle surface charge and possible charges on the bottle walls of both plastic and glass at the specific pH should be taken into account. Consequently, for engineered nanoparticles, adsorption to sample bottles needs to be investigated for both inorganic and carbon-based nanoparticles on a case-by-case basis until new experience-based knowledge has been accrued. Similar concerns apply to all other materials to which the sample is being exposed (e.g., tubing, filter materials, pipettes, amongst others).

Extracting inorganic nanoparticles from soil and sediment

Examining ENP in soils and sediments have the same limitations as for water samples, with the additional complication of much higher quantities of natural solids, many of which are in the same size range as the ENP. Dispersion methods for releasing natural nanomaterials from the solid matrix, such as sonication and chemical dispersants (hexametaphosphate, detergents, etc.) will likely release the ENP to the solution phase, but the physicochemical state of ENP will be likely to change (e.g., break-up of flocs). These protocols are reported in the soil literature (Gee and Bauder 1986). The separation of nanoparticles from soil suspensions or sediment slurries are difficult, and are prone to artifacts. As a general suggestion, centrifugation is generally less perturbing than filtration (Gimbert et al. 2005, 2006), but the differential settling during centrifugation can also induce aggregation. This is further discussed in the "Prefractionation" section below. The challenge then remains to discriminate between natural and engineered nanoparticles.

Extracting carbon-based nanoparticles from water, soil and sediment

Pristine fullerenes are comparatively soluble in organic solvents such as toluene and can be extracted from media (including water) into solvent (Fortner et al. 2005). In the case of CNTs (both single and multi-walled), Nowack and Bucheli (2007) summarise that no method currently exists for their quantification in natural media. Indeed, CNTs have low solubility, even in organic solvents.

Prefractionation

Environmental samples often contain complex mixtures of particles of different size classes, composition, shapes and are of biotic and/or abiotic origin. In order to study nanoparticles, it is often necessary to first reduce the complexity using a course prefractionation. The prefractionation can be based on settling, centrifugation or filtration. Settling or centrifugation is only effective in removing particles that have a settling velocity that dominates over their Brownian motion. The settling velocity depends on the particle volume, shape, and their density difference with respect to water. Therefore, settling or centrifugation is more efficient in removing more dense mineral particles than it is for algae and other organic particles. Centrifugation is a minimum perturbation prefractionation technique, but settling particles can scavenge other smaller particles due to the differential settling velocities.

Microfiltration, with pore sizes generally greater than 0.1 μm, is the most common prefractionation technique, due to its simplicity of operation. However, common "dead-end" filtration is prone to many artifacts, e.g., nanoparticle deposition, membrane concentration polarization, and filter cake formation (Buffle et al. 1992; Morrison and Benoit 2001).

Nanoparticles can be deposited on the membrane surface due to collision or electrostatic attraction. Particles smaller than the pore size can be transported through the membrane more slowly than the liquid, due to electrostatic repulsion within the pores. This causes concentration polarization (build up of higher particle concentration in the membranes diffusive boundary layer) which leads to

higher collision rates between particles and consequently aggregation. Aggregates or attached particles on the membranes, provides more efficient trapping of nanoparticles and their aggregates. This leads to formation of a filter cake and the effective pore size decreases severely; in other words the filter clogs.

These problems are especially severe for non-stabilized nanoparticles, e.g., those that lack hydrophilic surfaces. Therefore, filtration of engineered nanoparticle suspensions should be critically evaluated in terms of the scavenging of nanoparticles and, as a consequence, changing the size distribution.

Fractionation by ultrafiltration, nanofiltration and dialysis

Fractionation by membranes can either be done by applying a pressure to overcome the pressure drop across a membrane that sieves molecules or particles according to their size as in ultrafiltration or it can be done by letting solutes equilibrate across the membrane as in dialysis. The microfiltration artifacts mentioned above become greater as the pore size of the filter decreases (ultrafiltration and nanofiltration). This is especially critical where membranes are used as macromolecular sieves. In order to reduce the diffusive boundary layer over the membrane, and thereby minimize the concentration polarization over the membrane, cross-flow (or tangential) filtration (CFF) has been developed. In CFF the sample is recirculated (or stirred) in a reservoir on top of the membrane. A fraction of the sample with components smaller than the pore size, will pass through the membrane (to yield the permeate) in each cycle. By measuring the concentration of analyte in both the initial sample, the retentate (the fraction not passing through the membrane) and the permeate, it is possible to calculate the concentrations of analyte in the fractions smaller and larger than the membrane pore size. The performance of crossflow ultrafiltration has been extensively evaluated for natural colloids and reveals that the membrane type, membrane manufacturer, and operating conditions, have large influences on the fractionation results and recoveries obtained (Guo et al. 2000; Larsson et al. 2002; Liu and Lead 2006). Therefore, crossflow ultrafiltration should be appropriately tested and evaluated prior to application to ENPs. Ultrafiltration is a preparative size fractionation method that can be scaled to process large sample volumes and produce large quantities of isolated nanomaterials. Although it is limited to two fractions (above and below the membrane pore size), multi stage filtrations can allow for a crude size fractionation, however, this is extremely labor and time intensive. When the membrane pore size is below ~1 nm, the method is typically defined as nanofiltration. Nanofiltration is usually applied to the separation of molecules from salts and could potentially be applied to separate nanoparticles from their dissolved counterparts.

Dialysis is an ultra- or nanofiltration method that operates on diffusion of solutes across a membrane that arises from concentration gradients and osmotic pressure instead of pressure driven filtration (as is the case in CFF). Dialysis is a very mild fractionation method and it can be used to separate truly dissolved components (ions and small molecules) from their nanoparticle counterparts. Dialysis has been used to study nanoparticle-solute sorption behavior as well as nanoparticle dissolution, where the aqueous counterparts will diffuse across the dialysis membrane (Franklin et al. 2007). However, dialysis usually utilizes deionized or distilled water as an acceptor solution. This may promote dissolution or ionic strength changes which will lead to changes in dispersion state.

Field-flow fractionation, size exclusion and hydrodynamic chromatography

Field-Flow Fractionation (FFF) is a mild chromatography-like size-fractionating method that differs from chromatography in that it does not utilize a stationary phase. The most common FFF sub-technique is Flow FFF, which is discussed here. Flow FFF separates nanoparticles according to their particle size by virtue of their diffusion coefficients in a very thin open channel (Giddings 1993; Hassellöv et al. 2007; Schimpf et al. 2000). The separation principle relies on the combination of an applied field and longitudinal carrier flow. The field acts perpendicular to the length of the separation channel and causes the nanoparticles to move towards the accumulation wall. Nanoparticles form a cloud whose thickness is given by the particles' ability to oppose (generally through diffusion) the force of the field. Smaller particles will not be affected to the same extent as larger particles, and hence the smaller particles elevate higher in the channel. Perpendicular to the field, along the channel, the laminar separation flow is acting on the nanoparticles. The parabolic shape of the laminar flow velocity in the channel implies that particles traveling nearer to the middle of the channel move faster than particles traveling closer to the channel walls. Consequently, the smaller particles, having higher extending clouds, on average, travel faster than the larger particles, resulting in fractionation of the sample that provides a continuous size distribution. To monitor the size distributions, the FFF needs to be coupled to a detector that responds to the nanoparticle number or mass concentration. Examples include: UV absorbance, light scattering (von der Kammer et al. 2005b), or elemental detectors such as

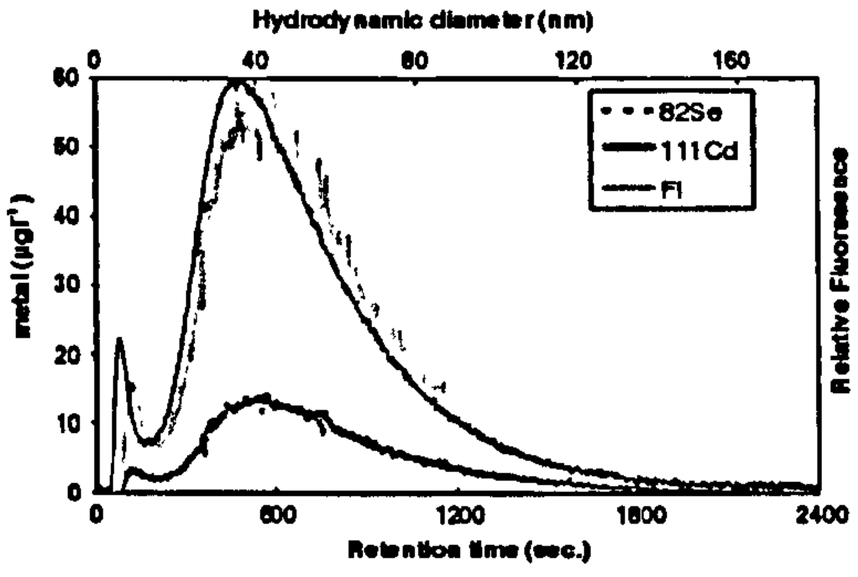

Fig. 2 Representative FFF fractogram of a CdSe quantum dot using on-line fluorescence and ICP-MS detection

ICP-MS (Hassellöv et al. 1999; Ranville et al. 1999; Jackson et al. 2005). The latter detector is very useful for characterizing metal-containing nanoparticles, an example being given in Fig. 2. Depending on the type of detector used, different kinds of size dependant information of the sample is achieved. One great advantage with FFF, compared to other fractionation methods, is that the retention time is directly proportional to nanoparticle physical properties. Retention in FFF is expressed as the retention ratio (R) given by

$$R = \frac{t^0}{t_r} \tag{1}$$

where t^0 is the void time and t_r is the sample retention time. For highly retained components, R can be approximated by

$$R \approx 6\lambda \tag{2}$$

while R can be estimated as follows for intermediate retention

$$R = 6\lambda\left[\coth\left(\frac{1}{2\lambda}\right) - 2\lambda\right] \tag{3}$$

The fundamental retention parameter (λ) is defined as the mean distance of the component from the wall (l) divided by the channel thickness (w).

$$\lambda = \frac{l}{w} = \frac{D}{Uw} \tag{4}$$

Channel thickness is calculated from experimentally determined channel volumes, since the actual channel thickness may differ from the manufacturer's specifications. Estimates of λ from experimental determinations of R allow calculation of the diffusion coefficient (D). It is important to note that the fundamental measurement made by Flow FFF is the diffusion coefficient. In the techniques of Flow FFF, diffusion coefficients can be used to determine hydrodynamic diameter. In Sedimentation FFF buoyant mass or equivalent spherical diameter can be determined (Giddings 1993).

The most critical factor in Flow FFF analysis is the choice of membrane and the carrier composition optimization. The particles should travel through the fractionation channel in close vicinity to the membrane without aggregating, adsorbing to the membrane or having inter-particle repulsion. This is generally accomplished for the complex natural samples by controlling the electrostatic repulsion and steric stabilization by a combination of suitable ionic strength (typically 0–20 mM monovalent salt) and a surfactant (e.g., 0.05% sodium dodecyl sulphate) (Hassellöv et al. 2007). FFF has been successfully applied to a wide range of synthetic nanoparticles (e.g., SiO_2, TiO_2, ZrO_2, Au, Ag, carbon black, pigments, Teflon, carbon nanotubes, soot particles) (Schimpf et al. 2000).

Another size fractionation method is size exclusion chromatography (SEC) where a particle or macromolecule mixture is passed through a column with a porous packing material with a distribution of pore sizes in the range of particles to be fractionated (Barth and Boyes 1992). The particles are separated according to their hydrodynamic volume (size and shape) by their ability to enter the porous structure of the packing materials. Particles that are larger enter pores to a lesser extent than the smaller particles. Each SEC column has a certain operating size (or molar mass) window, and the first eluting larger particles (all at once) are those outside the operating window, then come the fractionated particles and then the "salt peak" ions and molecules that have passed through the complete pore volume. Size exclusion chromatography has been applied to both carbon nanotubes and fullerenes, as described in a later section, to natural organic and inorganic nanomaterials (Perminova et al. 2003; Vogl and Heumann 1997; Jackson et al. 2005).

Hydrodynamic chromatography (HDC) is another size fractionation method that is carried out in narrow open capillaries, or in wider capillaries with non-porous packing materials that essentially form capillary routes. Due to the size, the center of mass cannot approach the walls infinitely and therefore a smaller particle can approach the wall to a larger extent than can a large one. Therefore, the elution order is the same as in SEC and also in the steric mode of FFF. The separation efficiency of HDC is very poor, but the operating size range is very good. HDC has been successfully applied for the fractionation of nanoparticles (Williams et al. 2002; Tiede unpublished results).

Chromatographic analyses of carbon nanoparticles

Many conventional techniques have been used to analyse fullerene solutions including UV–vis spectrophotometry,

infrared spectroscopy, nuclear magnetic resonance, and mass spectrometry, frequently coupled to high performance liquid chromatography (HPLC) (Andrievsky et al. 2002; Fortner et al. 2005; Isaacson et al. 2007; Nowack and Bucheli 2007; Treubig and Brown 2002). For HPLC, octadecyl silane (ODS) stationary phases are most commonly selected with elution using solvents such as toluene or toluene:acetonitrile mixtures (Treubig and Brown 2002). When UV–vis absorbance detection is used, 325 nm is the wavelength typically selected. Alternatively, gel permeation chromatography can be used, for example using Agilent PL gel 10 μm 50 A with toluene elution (Readman and Frickers, unpublished data). Size exclusion chromatography has also been applied to characterise CNTs (Duesberg et al. 1998).

Light scattering techniques

Light scattering is a very commonly used method to determine particle size (Schurtenberger and Newman 1993). The electromagnetic radiation of the incident photons induces an oscillating dipole in the particle electron cloud. As the dipole changes, electromagnetic radiation is scattered in all directions. The light source could be laser light, X-rays or neutrons, each of which enables probing at different size ranges and particle compositions. Discussion will mainly be limited to describing methods utilizing laser light, since these are the most readily available methods to be used in particle characterization for ecotoxicology.

Dynamic light scattering

In dynamic light scattering (DLS), also called photon correlation spectroscopy or quasielastic light scattering, fluctuations in the scattered light that depend on particle diffusion is utilized. The fluctuations originate from the Brownian motion of the particles and from the fact that neighboring particles can have constructive or destructive interference of the scattered light intensity in a certain direction. In the DLS instrument the intensity is measured over very short time periods (δt) and then it is possible to compare (correlate) the intensity at time t0 with time $t0 + \delta t$ (in the order of micro-milliseconds). Smaller particles (with faster diffusion) lose the correlation (the memory of their previous position) more rapidly than larger particles. The scattering intensity is plotted as an autocorrelation function:

$$g(\tau) = \left|G(\tau) - \langle I\rangle^2/\gamma\right|^{1/2} = Ae^{-2\Gamma\tau} \quad (5)$$

where $G(\tau)$ is the field autocorrelation function, $\langle I\rangle^2$ is the base line and γ is the coherence factor, expressing the efficiency of the photon collection. A is an instrument-specific constant, Γ is the decay rate and τ the delay time. Γ can be converted to the diffusion coefficient, D, using the relation:

$$D = \Gamma/q^2 \quad (6)$$

where q is the wave vector, which can be described by the following relation:

$$q = 4\pi\eta \sin(\pi/4)/\lambda \quad (7)$$

where η is the refractive index of the solvent and λ is the wavelength of the incident light. If the diffusion coefficient is known, the hydrodynamic radius, R_h, can be calculated from the Stokes–Einstein equation:

$$R_h = kT/6\pi\eta D \quad (8)$$

where k is Bolzmann's constant and T is the absolute temperature.

The advantages of DLS are: the rapid and simple operation, readily available equipment, and minimum perturbation of the sample (Ledin et al. 1994). The limitations are the interpretation, especially for polydisperse systems, and critical review of the data obtained (Filella et al. 1997). DLS gives an intensity weighted correlation function that can be converted to an intensity weighted (z-average) diffusion coefficient.

For $d < \lambda/20$, then the scattering intensity, $I \sim d^6$, according to the Rayleigh approximation, while for $\lambda/20 < d > \sim\lambda$ then $I \sim d^2$ (Debye approximation). The strong particle size dependence of the scattering intensity will bias the measured size, as a small amount of large particles will have such a large influence that smaller particles will be neglected. Consider a sample with two particle sizes, d: 3 and 30 nm, of equal particle number concentrations. The volume concentration will be 1,000 times larger in the 30 nm particles due to the geometrical formula of a sphere, but according to the Rayleigh approximation, the scattering intensity will be 10^6 times stronger for the 30 nm particle compared to the 3 nm particle. For even larger particles, the response difference will be enormous. Consequently, even the smallest fraction of dust or other micrometer-sized particles will ruin the signal from the nanoparticles.

For multimodal size distributions (multi component mixtures), the conversion of the autocorrelation function to diffusion coefficient is an ill-posed mathematical problem, where small variations can give large deviations in the output. For this reason, but more importantly due to the fact that the signal from larger particles dominates over smaller ones, a general rule is that DLS is not suitable for samples with polydispersity index above ~1.5–1.7.

Since DLS measures diffusion coefficients, and that all size calculations are based on assumptions that the Stokes–Einstein relation (Eq. 8) holds, it is essential to validate that

the diffusion coefficient measured is the undisturbed self-diffusion coefficient. For charged nanoparticles, electrostatic forces between particles have an effect on the diffusive behavior. This effect is concentration dependant, and the upper boundary occurs when the nanoparticle gets entrapped by forces from their close neighbors, the point of so-called gel-formation. By dilution of the sample to the greatest possible extent, while remaining above the detection limit, and extrapolation of the measured diffusion coefficient to infinite dilution, the unperturbed diffusion coefficient can be estimated. This value is one that can most reliably be used to calculate size in the Stoke Einstein equation. However, dilution of a sample will change its diffusion behavior and aggregation state. If primary particle size is not the goal, but rather to characterize the dispersion state in a sample, then it is more relevant to not dilute the sample, reporting diffusion coefficients only, rather than size.

It should also be noted that the derived data from DLS are intensity based distributions or averages, and mathematical conversions to volume or number distributions should only be provided with good knowledge of the particle shapes, polydispersity and underlying assumptions (Finsy 1994). Although dynamic light scattering does not provide full characterization of nanoparticle dispersion, it is very valuable to, for example, monitor aggregation behavior.

Static light scattering

Static light scattering (SLS), also called multi angle (laser) light scattering (MALS or MALLS), provides measurement of physical properties that are derived from the angular dependency of light scattered by a particle. This is due to the fact that a particle of a certain size generates destructive and constructive interferences at certain angles. Time averaged scattering intensities are measured at several angles to derive any number of several size parameters including the particle size, root mean square radius of gyration (R_g.), which is the root mean square distance of point masses in a particle from its center of gravity. Consequently SLS relates to the particle structure and morphology and can therefore be used in combination with DLS to give information of particle shape factors. There are several important assumptions in SLS theory for different analytical solutions. The most used is called Rayleigh–Gans–Debye approximation (Schurtenberger and Newman 1993). For these approximations, the refractive index difference between the particle and solvent should be negligible, the concentration of particles approaches zero, and no light absorption by the particles occurs.

Both dynamic and static light scattering polydisperse samples impose limitations on these methods. Therefore, it has been shown to be beneficial to couple light scattering detectors online to a fractionation method such as FFF or SEC (von der Kammer et al. 2005b; Wyatt 1998). With this combination, independent size distributions can be derived from the two methods and thereby, from comparison of the two results, distributions of particle shape factors can be estimated (von der Kammer 2005).

Nephelometry

Turbidity, or nephelometry, is a particle concentration measurement that utilizes scattering of light at 90° or sometimes 180°, with respect to the light source. The light source can be a laser or monochromatic light. The equipment is very simple and can be portable or even in situ, but the relationship between the concentration and particle concentration is not trivial. The light scattering intensity is, as mentioned above, strongly dependent on particle size, and also on other parameters such as the refractive index difference between the particles and the suspension media. Therefore, in quantitative analysis, turbidity measurements should only be used for well-defined particles of fairly narrow size distributions and complemented by calibration with other techniques (e.g., gravimetry). For dispersed nanoparticles, turbidity is fairly insensitive, and is less suitable than for monitoring aggregation.

Nephelometry has also been used as a chromatographic particle concentration detector (von der Kammer et al. 2005a).

Laser induced breakdown detection

Laser induced breakdown detection (LIBD) is based on the fact that when a solid nanoparticle passes through the focal volume of a focused, pulsed laser, the power density required to induce breakdown of the dielectric properties of the water is lower than for pure water (Kim and Walther 2007). If the laser energy is correctly tuned, plasma formation will only occur when a nanoparticle passes through the focal volume of the optical cell. The plasma formation, or breakdown, is detected with either a piezo-electric crystal attached to the cuvette, or with a CCD camera synchronized with the laser pulse. The parameter measured is the breakdown probability (BP). Since BP for a given laser energy depends both on particle concentration and on size, it is necessary to elucidate both. The most common mode is to tune the laser pulse energy and measure the BP of the sample, and do the same for a set of calibration standards of known size at different concentrations. The BP for larger nanoparticles has a threshold (increased from zero probability) at lower laser energies than smaller nanoparticles. The BP-laser energy curves have different

slopes depending on the concentrations that are also given from the calibration standards.

The main advantage of LIBD is that it is extremely sensitive even to small nanoparticles with detection limits in the ppt (ng dm^{-3}) range. In fact, LIBD is so sensitive that most samples have to be diluted in order to not saturate the breakdown probabilities.

The main disadvantages are that LIBD cannot discriminate between different types of nanoparticles and even more seriously, that different nanoparticle compositions have different breakdown probabilities (instrument responses). Therefore it is not possible to use one set of calibration standards for different types of nanoparticles. LIBD is a specialized technique that is not yet commercially available.

Spectroscopic analysis and characterization

Certain classes of nanoparticles demonstrate strong fluorescence and this property is utilized in many fields such as medical imaging, immunoassays, photonics, amongst others (Bailey et al. 2004). Quantum dots (QDs) are composed of semi-conductor materials, for example CdSe, CdS, CdTe, and are highly fluorescent. These particles can be characterized by either their absorption or fluorescence emission spectra. The absorption spectra is broad over low wavelengths but displays a sharp peak, called the first exciton peak at the upper wavelength of the absorption spectra. This peak is generally in the order of 20–50 nm lower in wavelength than the emission peak. The position of this absorption peak can be correlated to the particle size and is commonly used to monitor size in QD synthesis (Yu et al. 2003). The emission peak tends to be fairly narrow, on the order of 50 nm, with the wavelength being highly sensitive to nanoparticle size. Measurement of fluorescence spectra can thus also be used to determine particle size. In natural systems, natural fluorophores contained in humic substances and biological cells may interfere with these determinations. Non-fluorescent nanoparticles such as silica can be labeled with dyes to impart fluorescence. In some cases the fluorescence of the dye can be enhanced by the presence of a second dye that can contribute its exciton energy through a radiation-less transfer.

Quantitation of particle concentrations can be performed using absorption or fluorescence if the optical constants of the particles are known. For example, extinction coefficients for the first exciton peak of some QDs were determined by Yu et al. (2003). It is yet to be determined how significantly background absorption from natural occurring materials in water will limit the usefulness of UV–vis absorption for nanoparticle quantitation in aquatic systems.

Both UV–vis absorption and fluorescence can be used as online detectors for chromatography and FFF systems. The extremely bright fluorescence of some nanoparticles should provide low detection limits for these techniques. Figure 2 shows an example of the use of online fluorescence detection with FFF for a CdSe quantum dot.

Fluorescence microscopy gives spatial information and has been very useful in looking at the distribution of nanoparticles in cells and organisms. For example, uptake of QDs into the guts of filter feeding organisms is clearly observable using fluorescence microscopy.

For naturally fluorescent material or labeled macromolecules, fluorescence correlation spectroscopy within the focal point of a laser confocal microscope, have been successfully applied to determine the diffusion coefficients of these materials (Lead et al. 2000b). The principle is similar to dynamic light scattering (also called photon correlation spectroscopy), but the sensitivity is much better for small (fluorescent) particles. The method should be very suitable for studies of QDs in environmental media.

In describing the UV–vis absorption spectra of metal NPs, the term surface plasmon is used, which describes the oscillating electron clouds present at the metal-solution interface. Particle size strongly affects the absorption spectra through quantum confinement effects that are important at the nanometer scale of materials. The smaller the particle size, the lower the wavelength of light absorbed. Aggregation of NP results in band broadening and red shifting of surface plasmon band and has been used to study the effect of electrolytes on metal NP stability (Aryal et al. 2006).

Particle shape characterization of metal NP is also possible from examination of surface plasmons. While spherical gold and silver NP have strong surface plasmon bands at about 520 and 400 nm respectively, nanorods of these metals show two bands, a red-shifted long-axis band and a blue shifted short-axis band. The wavelength of the long axis band is particularly sensitive to particle aspect ratio. It has also been noted that Au nanorods have 106 stronger fluorescence than spherical Au NPs (Link and El-Sayed 1999). Consequently, surface plasmon effects can be used to study particle-particle interactions since the aspect ratio changes when to single particles come close together.

Electron microscopy and atomic force microscopy

There are several powerful microscopy techniques that can provide images of nanoparticle systems as well as additional information on elemental composition, structure and even charges or force measurements. Microscopy methods are all single particle methods, that is the data does not arise from an ensemble of particles such as is the case with

light scattering. This enables information to be collected on each particle free from interferences from other particles or background solutes. This gives good information on particle processes that sometimes cannot be obtained with bulk analysis (Mavrocordatos et al. 2007). However, it also means that even though a quantitative measurement with sometimes fairly good accuracy can be achieved on a single particle, it is only by counting and measuring enough particles (of a certain type or in a certain size range) that good enough counting statistics of the complete sample can be obtained. This is needed in order to deliver a quantitative analysis or characterization of the sample. Sizing with microscopy means that an average size measured on a certain number of particles are a number average, and in order to measure an accurate size distribution of nanoparticles it is necessary to count and measure thousands of particles in order to obtain a reliable counting statistics of the very few larger nanoparticles in the size distribution. The large particles in the distributions (or aggregates), even if very few, can contribute substantially to the volume or mass based distributions. In nanotechnology or material science this is not a problem since the particles to be measured are of the same type and are of similar size but when dispersed in water and mixed with natural organic matter and natural nanoparticles it is another story. Therefore we see a big need for automation in electron microscopy and development of "smart" image analysis software that enables characterization of the millions of particles needed in each sample (Mavrocordatos et al. 2007). With this said microscopy methods are very powerful for imaging and process understanding but it should be complemented with a particle population method that is giving quantitative information on the sample.

Another common feature for all microscopy techniques is that they require different levels of sample preparation. It ranges from the mildest being drying of the particles to a moist condition (AFM and ESEM) to a high-vacuum in SEM and TEM. In some methods coating or staining the sample is used. The transfer of the sample from its dispersed hydrated state to a dried high vacuum state often means that the particle size distribution changes dramatically. For example by evaporating a sample drop into dryness (a common method) the particle concentration and solute concentration increases drastically in the decreasing volume of the drop before it finally evaporates. This leads to aggregation of particles and precipitation of salts. Some methods are used to preserve the hydrated state of particles either by cryofixation, which is a rapid freezing so that the water forms non-crystalline ice. Another method is embedding the particles in some water-soluble resin that fixes the water when it cures.

The three most common sample preparation methods for natural colloids is drop deposition, adsorption deposition or ultracentrifugation harvesting, and the methods have been compared for AFM and electron microscopy respectively (Balnois and Wilkinson 2002; Mavrocordatos et al. 2007).

Scanning electron microscopy

In the family of electron microscopy techniques the sample is exposed to a high energy focused beam of electrons. In scanning electron microscopy (SEM) the interaction of the beam with the particle surface are scanned over the sample and measured as secondary electrons (most common), or backscattered electrons or X-ray photons. Due to the high depth of field in SEM a three dimensional appearance can be obtained. The sample needs to be conductively coated with gold or graphite and maintained under ultrahigh vacuum in order not to have the secondary electrons interact with gas molecules. The substrate is typically a filter membrane or a conducting grid.

Environmental scanning electron microscopy and related techniques

Due to the problems with morphological changes of the particles associated with the transfer to high vacuum state, environmental scanning electron microscopy (ESEM) was developed, where the sample cell is separated from the detector cell. This allows the sample to be measured under variable pressure and humidity (in theory up to 100%) with residual hydration water still on the particles. This water layer also serves as a conductor on the surface so the sample does not need to be conductively coated. The resolution is decreased (from ~10 to ~100 nm) due to the interactions of the secondary electrons with the water vapor molecules but there are less sample artifacts for example from natural colloids (Doucet et al. 2005). ESEM still allows analysis of the emitted X-rays. Wet STEM is a method for scanning TEM analysis of a wet sample on a TEM grid in an ESEM microscope utilizing dark-field imaging conditions with a resolution of a few tenths of nm (Bogner et al. 2005). A new sample capsule (WetSEM™) with electron transparent membranes provides an alternative to ESEM in ordinary SEM microscopes. The WetSEM capsules allow imaging under liquid or moist conditions (Thiberge et al. 2004). However, the loss of resolution is considerable (partly due to diffusion of the particles), and the membrane is sensitive to radiation damage, and only particles close to the membranes are in focus.

Transmission electron microscopy

In transmission electron microscopy (TEM) the electron beam is transmitted through a very thin specimen on a conducting grid (e.g., copper grid with a thin resin,

e.g., formvar). After the beam has been transmitted through the sample and has interacted with the particles the non-absorbed electrons are focused onto an imaging detector (fluorescence screen or CCD camera). In TEM the particles are shined through by the electron beam and the absorbance (image contrast) is both a function of the electron density of the elements in a particle and the thickness of the particles. Organic matter with only light elements needs to be stained by a heavy metal cocktail in order to be visible.

High-resolution TEM is a method that can give subnanometer resolution and is used in material science to study atom-by-atom structure. HR-TEM is a very demanding and time-consuming method but it has been applied to detect nanoparticle formation by bacteria or in geochemical processes (Banfield and Navrotsky 2001; Suzuki et al. 2002).

TEM has also been applied to characterize carbon nanoparticle dispersions in ecotoxicological exposure experiments (Smith et al. 2007).

Electron microscopy microanalysis

For all electron microscopy methods mentioned here analysis of spectral patterns of emitted X-rays (K, L & M lines) for elemental composition of the particles can be utilized if the microscopes are fitted with an energy dispersive X-ray spectrometer (EDX or sometimes EDS). The spatial resolution can be even less than10 nm. The sensitivity is best for heavier elements, so in reality it works best for major elements of the particles and associated heavy metals in fairly high concentrations. The measurement uncertainty of EDX is generally ~20% (Mavrocordatos et al. 2004, 2007).

Electron energy loss spectrometry (EELS) is another elemental composition method that can be applied in either spectrometric mode or in imaging mode in TEM. In EELS the loss of energies due to inelastic scattering processes (e.g., inner shell ionizations) can be interpreted to which elements that were causing the scattering. The energies lost are specific for each element. The EELS results are more difficult to interpret than EDX and works best for the lighter elements (from carbon and up to zinc). EELS can also be used to obtain additional chemical information (e.g., redox states of transition metals).

Atomic force microscopy

Atomic Force Microscopy (AFM) is a subnanometer resolution method in the family of scanning probe microscopy. It utilizes a cantilever with a very thin tip (tens of nm), that is oscillating over the surface of the sample. The oscillating movement (Z-axis) and the scanning over the surface (X and Y-axis) is controlled by piezoelectric actuators.

A laser-based balance can measure both repulsive (Pauli principle) and attractive (van der Waals) forces between the tip and the sample in the range 10^{-7} to 10^{-12} N. The occurrence of these forces at different stages of the cantilever oscillation can be used to derive a separating distance between the tip and the particles. The resulting images are an atomic force topography. The substrates that the particle samples are prepared on should be atomically flat (mica, graphite or silicon wafers are examples of suitable substrates). The preparation methods are typically drop deposition, adsorption deposition or ultracentrifugation as for electron microscopy, but in addition it is possible to analyze samples under moist conditions or even in liquids, which affords minimum perturbation. However, under liquid conditions the particles are sometimes attracted to the substrate (very weakly) and are moved around disturbing the images. Another feature in AFM is that the geometry of the tip compared to particle size gives that the tip is starting to "feel" the particle significantly before its center has approached the particle periphery, and analogously when the scanning tip is leaving the particle it feels the particle forces too long. Therefore the lateral dimensions are greatly overestimated, while the height measurements are very accurate. This should be kept in mind when interpreting AFM images, which means, e.g., a carbon nanotube can give a height of 1 nm but a width of up to 50 nm even though these should be the same. The geometry of the tip should be decreased if small particles are to be more accurately probed. The cantilever tip can be set to contact the particles but lateral forces lead to movement of particles, so a tapping mode or a non-contact mode has been developed to just feel the forces above the particles (Balnois et al. 2007). The latter has shown to be more accurate for soft, compressible particles such as humic acids. AFM is one of the most common nanometrology methods and has numerous applications (e.g., Lead et al. 2005; Viguie et al. 2007).

In Fig. 3, a dispersed ZnO nanopowder sample (with manufacturer stated size 50–70 nm) has been prepared with adsorption deposition and analyzed with AFM, TEM and SEM. The difference in visualization and size measurements is clear. AFM and TEM show sintered aggregates with primary particles in the size range provided by manufacturers, whilst SEM shows mainly larger flakes of material with some nanoparticles on top. It is likely that the sample preparation and vacuum-induced changes can explain these differences.

Surface charge measurements

Colloidal nanoparticles develop surface charges in aqueous solutions. The net surface charge, or surface potential, is

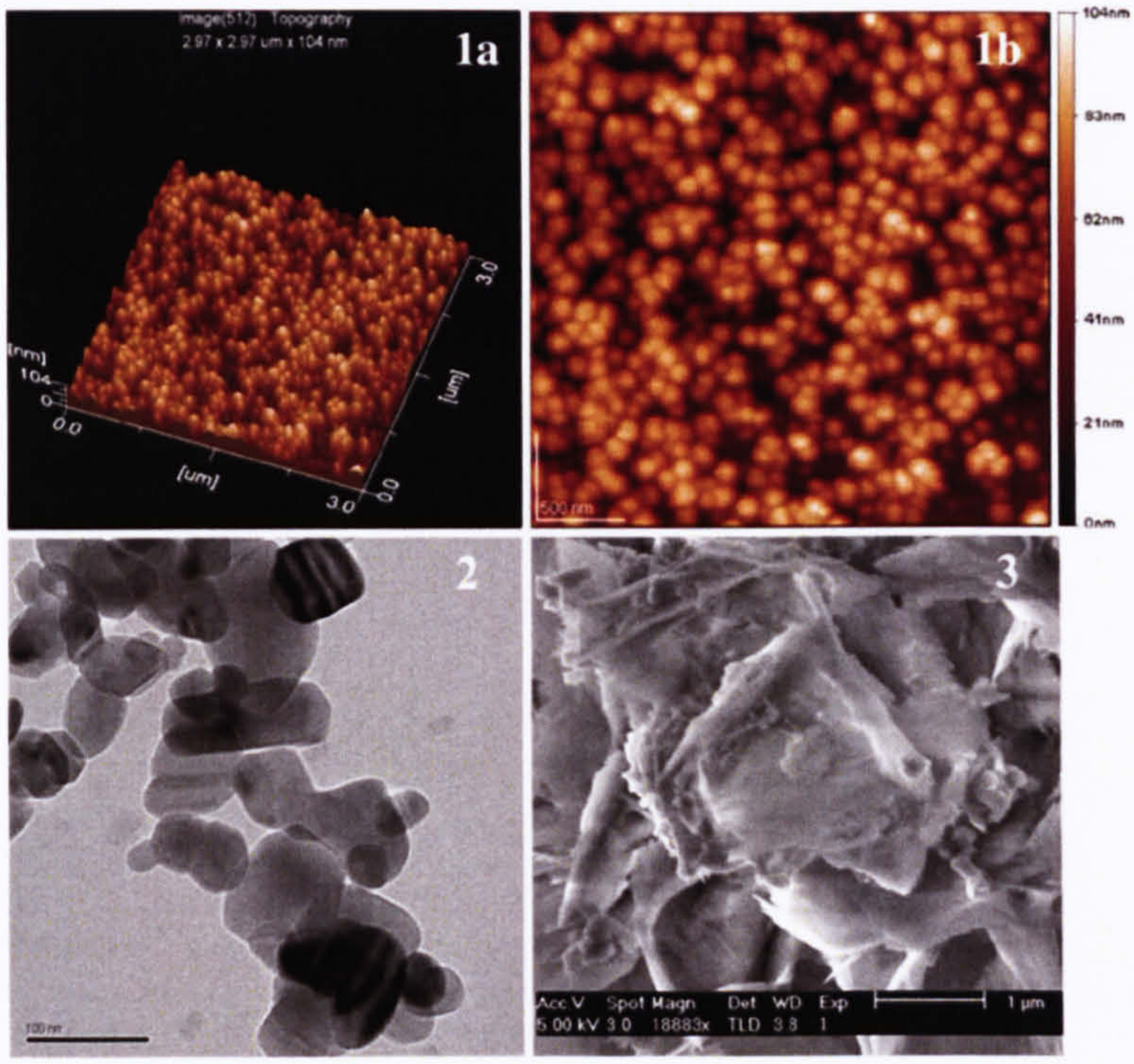

Fig. 3 ZnO nanoparticle powder (50–70 nm, Sigma Aldrich UK), dispersed in distilled water ($\sim$5 mg l^{-1}), allowed to dry on silica and imaged by AFM (1a and b), TEM (2) and SEM (3) under standard conditions

one of the most important nanoparticle characteristics since it describes to what extent the nanoparticle dispersion is electrostatically stabilized by interparticle repulsion. Consequently, ENP surface potential will have major influence on their fate and behavior (Guzman et al. 2006; Hunter and Liss 1979). However, it is not easy to directly measure the surface potential but there is a simple method that measures the so-called zeta potential, which is the potential at a hydrodynamic slipping plane in the electrostatic double layer of the particles as measured by electrophoresis. The measured electrophoretic mobility can be converted to z potential through Smoluchowski's theories. The point of zero charge (PZC) is the pH where negative and positive charges are balanced, so there is no net charge on the nanoparticles. At PZC there is generally maximum aggregation taking place since the particles are allowed to come in close contact so that attractive van der Waals forces can act.

Surface area measurement

The Brunauer, Emmett, Teller (BET) (Brunauer et al. 1938) method is used to measure the specific surface area of solids, which involves drying of a powder in vacuum and then measuring (using a microbalance) the adsorption of dinitrogen gas (assumed as a monolayer) on the surface and in micropores. The BET method builds on the assumption that N_2 has access to the complete surface of the particles. Other variants of this method based on adsorption of organic molecules (e.g., ethylene glycol monoethyl ether, EGME) can be used (Hassellöv et al. 2001). Dinitrogen gas gives higher surface areas than EGME, probably due to greater access to smaller pores.

Crystal structure

X-ray diffraction (XRD) is a method of measuring interparticle spacings resulting from interference between waves reflecting from different crystal planes. It is used in mineralogy to determine crystal structure of mineral particles. For example XRD can be used to distinguish between the anatase and rutile and amorphous phases of TiO_2 nanoparticles. A dry sample needs to be prepared as a thin film. Elemental composition of major elements can also be obtained although the sensitivity is low compared to other elemental analysis methods (e.g., ICPMS or AES).

It is also possible in TEM to measure the diffraction patterns of single particles using a method called "Selected area electron diffraction" (SAD, or SAED). In SAD the user can select an area of the sample with a small aperture and only the electron diffraction pattern from that area will be measured. This has benefits over XRD for heterogeneous samples because it allows single particle characterization.

Difference in analysis of particulate and nanoparticulate assemblages compared to conventional analysis of solutes

For analysis of nanoparticle assemblages by bulk analytical methods (in contrast to single particle analysis methods, e.g., microscopy) in whole samples or on fractions after sample treatment (e.g., filtration or Field-Flow Fractionation), it is necessary to recognize that for certain methods there may be differences compared to more common analysis of dissolved solutes (e.g., ions or molecules). In bulk analysis of a nanoparticle dispersion the analytes mass concentration are not homogeneously distributed, but rather as uniformly distributed point masses. This is not a problem providing the probed sample volume of the method is not approaching that of single nanoparticles. But when analyzing samples with environmentally relevant concentrations, with methods that are probing a very small samples volume (e.g., a very rapid measurement in a capillary or a fast flowing sample stream such as in mass spectrometers) the measurement may approach or enter a domain of single nanoparticle events. The consequence is a noisier signal and if there is statistically less than one particle per measurement then the recovery of the determination decreases, which gives an erroneous determination. Since the particle numbers (for the same mass) decreases rapidly for larger particles, this issue is more severe for them than for smaller particles. This is a well-known phenomena in e.g., ICPMS analysis of micrometer sized particles, and is called slurry nebulization. It needs to be considered when the number concentration is low. Other problems may be non-quantitative measurement of the particles, for example through incomplete atomization in elemental analyses or non-transparent or shading effects in spectroscopy. For slurry nebulization in ICP-AES or ICP-MS, it has been found that the particle size is the dominating factor to obtain complete atomization, where particles below 3–5 μm have been found to yield quantitative recoveries compared to solutions (Ebdon et al. 1997; Santos and Nobrega 2006). The main reason for decreasing recoveries was poor transport efficiencies in the nebulizer-spraychamber system. This implies that for nanoparticles, incomplete atomization should not be a problem but maybe for aggregates particularly refractory materials such as carbides and some oxides may also present problems.

Validation, measurement uncertainty and good laboratory practices

In metrology and analytical chemistry, it is fundamental to be able to report on the traceability of the acquired results. Calibration standards used for quantification are generally traceable to a primary national or international standard. However, for nanoparticles the validity of these standards has a shorter lifetime than most other standards and is more sensitive to operating conditions. Nanoparticle standards, or reference materials, exist both as suspensions and as powders. Nanoparticle standards in suspension are generally labeled with expiry dates and instructions for storage. Sometimes there are also instructions on how to further dilute the standard in order to maintain its integrity. The use of powdered nanoparticle standards does not include a standardized procedure for dispersion of the nanoparticles. To make the dispersion in each individual laboratory increases the uncertainty of the original metric stated by the manufacturer. Indeed, many metrics (e.g., size distribution) are strongly dependant on how the dispersion was made and in which media (pH, ionic strength and composition and presence of organic matter).

In addition to the nanometrology specific issues of method validation relates to the normal quality control (QC) of any analytical method (Table 5). The most important steps in analytical QC are method validation and quantification of measurement uncertainty. The method validation is simply an experimental procedure to determine that the method and procedures (standard or in-house developed) are complying with the documented specifications (e.g., limit of detection, linearity, determination of precision and accuracy and robustness). One way of determining the accuracy is to use a certified reference material (CRM) of the same type as the samples and with documented property values within the range of the method (Table 5). CRMs or NIST traceable size standards are however very rare for nanoparticles as yet. There exist reference materials with certified sizes for gold and polystyrene colloids in the nanometer size range. More reference materials are under development through international efforts. Testing the homogeneity, shelf life of a reference material and carrying out all the analysis in order to certify the material is very elaborate and expensive. In the absence of CRMs there is also the possibility to use non-certified materials to (test materials) to benchmark analytical procedures and toxicity testing (Aitken et al. 2007).

Another option is to participate in interlaboratory comparisons were a blind sample is sent to many laboratories for analysis, thereby affording a good indication of accuracy and precision in the results. Interlaboratory comparisons are not yet as common in nanometrology as in conventional analytical chemistry where rigorous quality assurance protocols are followed in order to achieve and maintain certified accreditation.

There are, however, a few examples of informal interlaboratory comparisons on natural nanoparticles (Lead et al. 2000a) and on engineered nanomaterials (Breil et al. 2002) which have proved highly informative to the participants and for other users of the same methodologies. A good daily routine is to analyze a QC sample and plot that value into a control diagram to monitor measurement uncertainty between interlaboratory comparisons. The QC sample should be a sample that is stable over time and that is as similar to the usual samples as possible. Thus can method or instrument related problems in the laboratory can be easily and quickly discovered.

Good laboratory practices in characterization of exposure/effect experiments should include minimal sample perturbation and determination of the dispersion-agglomeration state. Dynamic light scattering fulfills these criteria, is a simple measurement to perform, and is available in most academic institutions. It is also a simple measurement to perform. However, for the reasons described previously, the results from DLS should not be over interpreted. DLS is primarily not a size determination method as it measures scattering intensity weighted diffusion coefficients. Thus, it is well suited to follow initial stages of aggregation, but not to provide nanoparticle sizes. For toxicity tests of nanoparticles, we suggest to conduct a separate dispersion experiment under optimum conditions as a reference to the dispersion behavior in the effect media and during the course of the effect experiment. This reference experiment with maximum dispersion may include surfactants, co-solvents, certain ionic strength and sonication. By comparing the results in the realistic effect/exposure experiments with this reference experiment one can obtain information on the degree of aggregation.

If competence and equipment is available a less biased (but with slightly more perturbation) determination of the size distribution can be achieved using e.g., Field-Flow Fractionation. Microscopy (e.g., AFM, SEM or TEM) is very powerful in imaging nanoparticles and aggregates, but the aggregation state of the sample may have changed during sample preparation.

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