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## Development and validation of a system for the generation, characterization and subsequent air- liquid interface studies of aerosol particles

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2016

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*Citation for published version (APA):*

Svensson, C. (2016). *Development and validation of a system for the generation, characterization and subsequent air- liquid interface studies of aerosol particles*. [Doctoral Thesis (compilation), Ergonomics and Aerosol Technology].

*Total number of authors:*

1

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Development and validation of a  
system for the generation,  
characterization and subsequent air-  
liquid interface studies of aerosol  
particles

Christian R. Svensson

DOCTORAL DISSERTATION

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To be defended at Stora Hörsalen, IKDC, Lund. Date 2015-02-12 and time 10:15.

*Faculty opponent*

Otmar Schmid

Organization LUND UNIVERSITY Department of Design Sciences Division of Ergonomics and Aerosol Technology Author Christian R Svensson	Document name Doctoral Thesis
	Date of issue 2016-01-19
	Sponsoring organization
Title and subtitle: Development and validation of a system for the generation, characterization and subsequent air-liquid interface studies of aerosol particles	
<p>Abstract: Exposure to nano-sized and nanostructured aerosol particles with tailored properties are likely to increase in society. Occupational exposure to various kinds of nanostructured aerosol particles such as soot and metal aggregates already exists. To understand the adverse human health effects of these particles, there is a need for systems that can Generate a stable output of aerosol particles, Characterize their exposure and dose, and determine their toxicological and biological effects in an Air-liquid interface setting (GCA).</p> <p>The research presented in this thesis developed and validated a GCA that combines a high output of aerosol particles, online characterization, collection capabilities for a scanning electron microscope (SEM) and transmission electron microscopy (TEM), as well as wet samples for protein interaction studies and particle toxicological effects.</p> <p>The GCA is composed of an air-liquid interface cellular exposure chamber – the Nano Aerosol Chamber for In Vitro Toxicity (NACIVT) – and an electrostatic precipitator. It also includes a high output aerosol particle generator with built-in capabilities for sintering and online mass mobility characterization, a scanning mobility particle sizer, and a tapered element oscillating microbalance (TEOM) for continuous characteristics and exposure measurements.</p> <p>The output characteristics of two spark discharge generators and a high temperature evaporation furnace were determined, including mass mobility relation. The results show that all three of these generators produced a stable output of nanostructured metal aerosol particles in the 30-300 nm size range; when sintered, these were near spherical in shape. In terms of dose, number, mass and surface area, the output was comparable to that of previous studies for all generators.</p> <p>The GCA with respect to toxicological studies was validated using primary human small airway epithelial cells (SAEC) and carcinoma cell line (A549). Aerosol particles of copper (Cu), palladium (Pd) and silver (Ag) were generated and administered in the NACIVT during 1 hour exposures. For SAEC exposed to Cu, Pd or Ag, a significant (<math>p &lt; 0.05</math>) effect on both viability and cytokine expression was observed. Also a significant (<math>p &lt; 0.05</math>) dose response for SAEC exposed to Cu, Pd and Ag could be determined. For the A549, Cu and Pd exposure induced a significant reduction in cellular viability.</p> <p>The protein interactions of the near-spherical sintered and aggregated Au nanoparticles, that were generated using high temperature evaporation furnace, were investigated in serum and human lung fluid solutions. When administered to the air-liquid interface, the spherical Au particles formed a stable film with a pinkish hue; when agitated, &lt;100 nm aggregates of particles and proteins were formed. This film formation is suggested as a protective property of the air-liquid interface itself.</p>	
Key words: air liquid interface, nanoparticles, aggregates, aerosol, protein interactions, toxicology, dose	
Classification system and/or index terms (if any)	
Supplementary bibliographical information	Language: English
ISSN and key title 1650-9773 publication 55	ISBN 978-91-7623-622-2
Recipient's notes	Number of pages
	Price
	Security classification

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Faculty and Department  
ISBN 978-91-7623-622-2 (Print)  
ISBN 978-91-7623-623-9 (Pdf)  
ISSN 1650-9773 publication 55

Printed in Sweden by Media-Tryck, Lund University, Sweden  
Lund, 2015



KLIMATKOMPENSERAT  
PAPPER



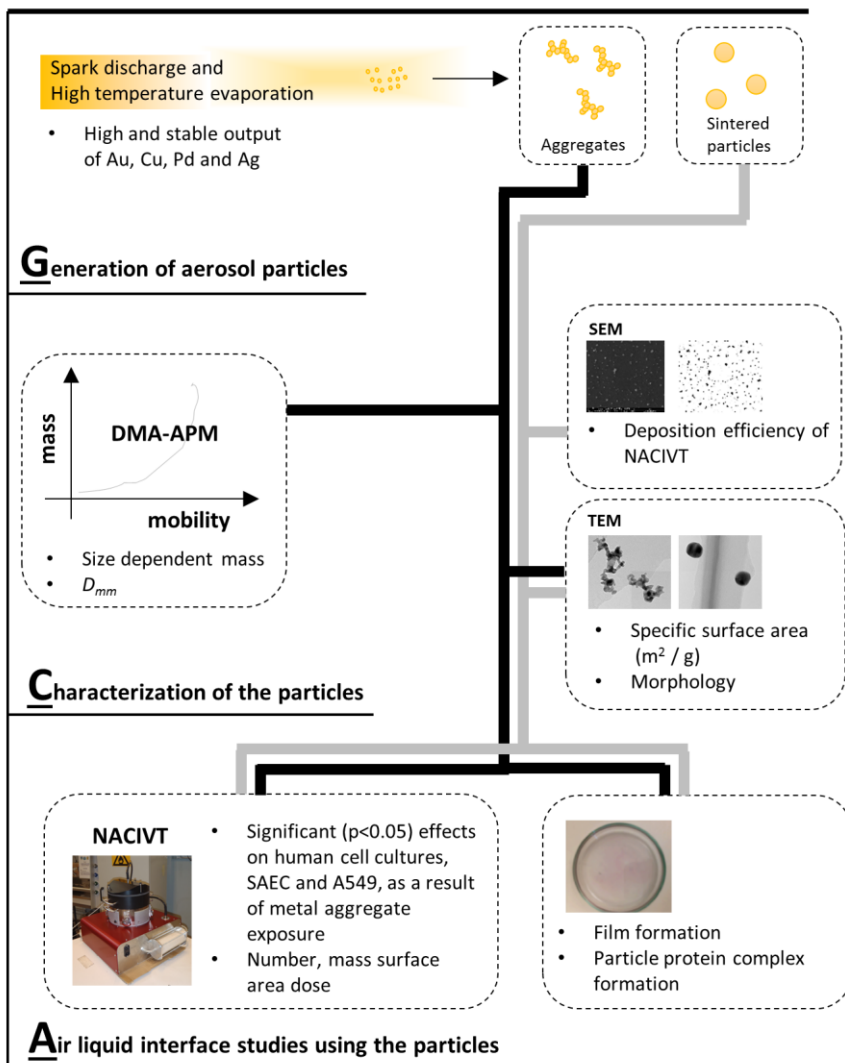
*“We are dreamers, shapers, singers and makers.”*

**Babylon 5**



# Graphical abstract

## Development and validation of a GCA







# Abstract

Exposure to nano-sized and nanostructured aerosol particles with tailored properties are likely to increase in society. Occupational exposure to various kinds of nanostructured aerosol particles such as soot and metal aggregates already exists. To understand the adverse human health effects of these particles, there is a need for systems that can Generate a stable output of aerosol particles, Characterize their exposure and dose, and determine their toxicological and biological effects in an Air-liquid interface setting (GCA).

The research presented in this thesis developed and validated a GCA that combines a high output of aerosol particles, online characterization, collection capabilities for a scanning electron microscope (SEM) and transmission electron microscopy (TEM), as well as wet samples for protein interaction studies and particle toxicological effects.

The GCA is composed of an air-liquid interface cellular exposure chamber – the Nano Aerosol Chamber for In Vitro Toxicity (NACIVT) – and an electrostatic precipitator. It also includes a high output aerosol particle generator with built-in capabilities for sintering and online mass mobility characterization, a scanning mobility particle sizer, and a tapered element oscillating microbalance (TEOM) for continuous characteristics and exposure measurements.

The output characteristics of two spark discharge generators and a high temperature evaporation furnace were determined, including mass mobility relation. The results show that all three of these generators produced a stable output of nanostructured metal aerosol particles in the 30-300 nm size range; when sintered, these were near spherical in shape. In terms of dose, number, mass and surface area, the output was comparable to that of previous studies for all generators.

The GCA with respect to toxicological studies was validated using primary human small airway epithelial cells (SAEC) and carcinoma cell line (A549). Aerosol particles of copper (Cu), palladium (Pd) and silver (Ag) were generated and administered in the NACIVT during 1 hour exposures. For SAEC exposed to Cu, Pd or Ag, a significant ( $p < 0.05$ ) effect on both viability and cytokine expression was observed. Also a significant ( $p < 0.05$ ) dose response for SAEC exposed to Cu, Pd and Ag could be determined. For the A549, Cu and Pd exposure induced a significant reduction in cellular viability.

The protein interactions of the near-spherical sintered and aggregated Au nanoparticles, that were generated using high temperature evaporation furnace, were investigated in serum and human lung fluid solutions. When administered to the air-liquid interface, the spherical Au particles formed a stable film with a pinkish hue; when agitated, <100 nm aggregates of particles and proteins were formed. This film formation is suggested as a protective property of the air-liquid interface itself.

# Populärvetenskaplig sammanfattning

Tänk dig ett system som kan bestämma hur olika typer av luftpartiklar skadar oss när dom hamnar i våra lungor. Ett system som dessutom kan skapa luftpartiklar för dessa studier vid behov och med väl uppmätta egenskaper. Denna avhandling handlar om just ett sådant system och experiment som är viktiga för att förstå om och hur det fungerar. Systemet kallas i denna avhandling GCA, från orden Generation, Characterization och Air-liquid interface.

Det har visat sig att människan kan ta stor skada av att inandas för många partiklar av så att säga fel slag. Man kan tänka sig olika typer av luftburna metallpartiklar, som bildas när man bearbetar metall, eller sotpartiklar från förbränning. Det uppfinns också hela tiden nya industriella processer och produkter, tänk bara på nanotekniken. Man kan säga att man vill förebygga olyckan, vi vill inte bara vänta och se vilka partiklar som är farliga och inte.

GCA som avhandlingen handlar om, tillhör en forskningstradition som fokuserar på att studera celler odlade utanför kroppen, så kallat in vitro forskning. Dessa cellkulturer av lungceller exponeras sedan för partiklar direkt från luften, precis som det sker i lungan. Denna typen av experiment kommer vara väldigt viktiga framgent då EU skärpt sina regler anngående djurförsök. Det är inte heller så populärt alltid att exponera människor direkt för något man misstänker är farligt.

GCA består av flera delar, förutom kammaren där cellerna blir exponerade för partiklar har det förmågan att bestämma partiklarnas storlek, massa, ytarea och antalskoncentration. Allt detta görs för att få en så klar bild som möjligt om vilken egenskap som partiklarna har som egentligen är farlig.

Resultat från GCA visar att det ger utslag när celler blir exponerade för koppar, silver eller palladium partiklar som från luftburet tillstånd landar på cellerna. Resultaten visar att man i vissa fall kan se att effekten på cellerna blir större med en ökad exponering. Avhandlingen beskriver också resultat från ett experiment som kan komma att vara väldigt viktigt för denna typ av framtida forskning. Experimentet visar att när vissa partiklar landar på en vätskeyta stannar dom där. Detta kan betyda att gränsskiktet mellan luft och vätska har vissa beskyddande egenskaper för underliggande celler.



# Acknowledgments

## **EAT & Nuclear Physics**

I would like to thank my main supervisor Mats Bohgard and supervisors Jenny Rissler, Joakim Pagels, Anders Gudmundsson and Tommy Cedervall. As a result of your commitment, concern and kindness my time at EAT has been as rewarding as pleasurable!

Thank you Patrik Nilsson, comrade and colleague for many a pleasant “fika”, for sharing ups and downs and superior technical assistance.

Thank you Christina Isaxon for friendship, many funny and interesting discussions and good science-times!

Thank you Aneta for your support and friendship. Your kindness and wisdom makes people confide and trust in you, great traits for a future research leader!

Thank you Erik Nordin for your friendship, assistance and discussions.

Thank you Andreas Dahl for helping me with LabVIEW and friendship.

Thank you Göran Frank for your friendship and helping me out in the lab many times.

Thank you Birgitta for your friendship and your participation with the LabVIEW course!

Thank you Erik Fors for being a fun friend and helping me out a lot!

Thank you Emelie for many fun fika times!

Thank you Eileen for making my thesis and papers readable!

Thank you the entire group working with aerosols and EAT! It is a good place to work and do research with much support and good spirits.

Thank you service-group and all administrative personnel at EAT, you have helped me out a lot many times!

## **FTF**

Thank you Maria Messing for being a good friend during this time as well as competent advisor. Also for talking me into the world of research.

Thank you Bengt Meuller and Linus Ludvigsson for friendship, technical assistance and many interesting discussions.

Thank you Team-Aeroid! Maria, Bengt, Linus and Julia Adner. Also thank you Anders Gudmundsson for helping Julia making the final perfect experiment!

Thank you Knut for your good spirits, they really do rub off!

### **CMPS**

Thank you Karin, Risto and Sara for friendship, help and interesting discussions!

Already mentioned you Tommy, but you sure have earned more thanks!

Thank you Martin for interesting discussions about particle measurements in solution.

### **AMM**

Thank you Shegufta, Karin, Monica, Ayman and Naserin for your good work and spirits on the air-liquid interface project! It was difficult but we made it, maybe even the paper is published as you read this!

Thank you all of AMM as you helped me weighing, drying and other activities during my first time as phd student.

### **Other Academia**

Thank you Bosse for helping us out with the labview course and being a fun person!

Thank you Linda Önnby for inviting me to participate in your study and being a co-author! It was so much fun!

Thank you John Janiak for helping me understand the DLS and for your friendship!

Thank you Elodie for your hard work with the air liquid interface project, without you it would never have worked out! Thanks also for being a good and funny friend!

Thank you Patrik Bauer and Per-Fredrik for being funny interesting comrades and colleagues!

Thank you Vincent Castranova for receiving me so well in Morgantown on my visit!

Thank you Ello for keeping things in perspective!

Thank you Otmar for being my opponent and thank you Betygsnämnd!

### **Life outside academia**

Thank you family for your support during this time. Mother Rose-Marie, brother Christoffer and sister Anna!

Thank you dad!

Thank you morfar and mormor, I am sure you are out there somewhere somehow.

Thank you other family members, Micke, Cina, Marcus, farmor, farfar among many others!

Thank you friends from Karlskrona, Patrik, Johan, Henrik, Fredrik, Jonas, among many others.

Thank you new friends of Skåne, Sweden and the world.





# Symbols and abbreviations

A549	Lung carcinoma cell line
ALICE	Air-Liquid Interface Cell Exposure system
APM	Aerosol particle mass analyzer
APS	Aerodynamic particle sizer
BSA	Bovine serum albumin
CB	Carbon black
CMD	Count median diameter
CPC	Condensation particle counter
$D$	Fractal dimension
$d_{ae}$	Aerodynamic diameter
DLS	Dynamic light scattering
DMA	Differential mobility analyzer
$d_{me}$	Electrical mobility diameter
$D_{mm}$	Mass mobility exponent
EU	European Union
EURL ECVAM	EU Reference Laboratory for Alternatives to Animal Testing
GCA	Generation, Characterization and Air-liquid interface
HT	High temperature evaporation furnace
$k$	Pre factor in power law, K-factor
$m$	Mass
$N$	Primary particle number
NACIVT	Nano Aerosol Chamber for In Vitro Toxicity
NTA	Nanoparticle tracking analysis

PM	Particulate matter
$q$	Elemental charge
$r_i$	Radius relevant for APM
$r_g$	Radius of gyration
SAEC	Small airway epithelial cells
SDG <sub>C</sub>	Spark discharge generator developed at FTF, LU
SDG <sub>P</sub>	Spark discharge generator (Palas GMBH)
SDS PAGE	Sodium dodecylsulphate polyacrylamide gel electroforesis
SEM	Scanning electron microscope
SMPS	Scanning mobility particle sizer
SSA	Specific surface area
TEM	Transmission electron microscopy
TEOM	Tapered element oscillating microbalance
UFP	Ultra fine particles
$\rho_{eff}$	Effective density
$\chi$	Shape factor
$\omega$	Angular velocity

# List of papers included in thesis

- I. Messing, M. E., **Svensson, C. R.**, Pagels, J., Mueller, B. O., Deppert, K., & Rissler, J. (2012). Gas-borne particles with tunable and highly controlled characteristics for nanotoxicology studies. *Nanotoxicology*, 7(6), 1052-1063.
- II. **Svensson, C. R.**, Messing, M. E., Lundqvist, M., Schollin, A., Deppert, K., Pagels, J. H., Rissler, J., & Cedervall, T. (2013). Direct deposition of gas phase generated aerosol gold nanoparticles into biological fluids-corona formation and particle size shifts. *PLoS one*, 8(9), e74702.
- III. **Svensson, C. R.**, Ludvigsson, L., Mueller, B. O., Eggersdorfer, M. L., Deppert, K., Bohgard, M., Pagels, J. H., Messing, M. E., & Rissler, J. (2015). Characteristics of airborne gold aggregates generated by spark discharge and high temperature evaporation furnace: Mass-mobility relationship and surface area. *Journal of Aerosol Science*, 87, 38-52.
- IV. **Svensson, C. R.**, Ameer, S. S., Ludvigsson, L., Ali, N., Alhmdow, A., Messing, M. E., Pagels, J.H., Gudmundsson, A., Bohgard, M., Sanfins, E., Broberg Palmgren, K., Kårdedal, M., & Rissler, J. (2015). Validation of an air-liquid interface toxicological set-up using Cu, Pd and Ag well-characterized, nanostructured aggregates and spheres. *Submitted for publication*.



# Author's contributions to the papers

- I. I participated in the experiment design and execution, performed the data analysis and produced the figures for publication. The dataset was generated during my time as a Master's student at the Division of Ergonomics and Aerosol Technology, Lund University.
- II. I participated in the experiment design, execution and data analysis. A large portion of the analysis dealt with understanding the techniques of size determination of nanoparticles in solution. I was the lead person in writing the manuscript.
- III. Data from Paper I and new data were used to determine the set aerosol surface area content and mass mobility characteristics. I participated in the study design, experimentation and performed the data analysis. I was the lead person in writing the manuscript.
- IV. I participated in the study design and developed the aerosol portion of the experimental set-up. I operated the cell exposure device and performed the particle characterization, determination of deposition efficiency and compiled the results from the toxicological analysis. I was the lead person in writing the manuscript.



# Papers not included in thesis

- i. Önnby, L., **Svensson, C.**, Mbundi, L., Busquets, R., Cundy, A., & Kirsebom, H. (2014).  $\gamma$ -Al<sub>2</sub>O<sub>3</sub>-based nanocomposite adsorbents for arsenic (V) removal: Assessing performance, toxicity and particle leakage. *Science of the Total Environment*, 473, 207-214.
- ii. Ali, N., Mattsson, K., Rissler, J., Karlsson, H. M., **Svensson, C. R.**, Gudmundsson, A., Lindh, C. H., Jönsson, B. A., Cedervall, T., & Kåredal, M. (2015). Analysis of nanoparticle–protein coronas formed in vitro between nanosized welding particles and nasal lavage proteins. *Nanotoxicology*, 1-9.

For a complete list of conference papers and contributions please visit:

<https://lup.lub.lu.se/search/person/desi-cts>





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# Background

We are constantly exposed to airborne solid or aqueous particulates. This thesis is devoted to the study of how these airborne particles can be characterized with regards to size, mass, biological interaction and toxicology. “Why do we need to care?” you ask. Let me set the stage.

When it comes to urban pollution, an example often used of the human health effects of particles is the London fog of 1952 (Wilkins, 1954). This study found that the increase in airborne particulate matter (PM) mass concentration was positively related to the increase in mortality. In the Harvard six U.S. cities study, an increase in urban PM was significantly associated with an increase in mortality rates (Dockery et al., 1993). The World Health Organization, through the International Agency for Research on Cancer, has concluded that diesel engine exhaust is a cancerogen to humans (Benbrahim-Tallaa et al., 2012). Pollution can also penetrate into our homes (Vette et al., 2001), and it has been shown that our own indoor activities contribute to indoor PM (Isaxon et al., 2015).

In an industrial setting, exposure to welding fumes, PM and various gases, have been linked to numerous adverse health effects as summarized in Antonini et al. (2003). Occupational exposure to mineral dust is well known to be associated with the onset of silicosis (Leung et al., 2012). Several conferences during the 20<sup>th</sup> century, the first one organized in Johannesburg South Africa in 1930, explored and described the cause and effect of silicosis and mineral PM. To this day, cases of lung cancer are reported as a result of asbestos exposure, a long persistent fiber-type particle (Hodgson & Darnton, 2000).

Of special interest is the exposure to and potential toxic effects of engineered nanoparticles. Various types of nanosized carbon, often referred to as carbon black (CB), are used in coloring agents and in many other applications (Kuhlbusch et al., 2004). Titanium dioxide, in various size regimes, has long been produced for use as a coloring agent, food additive and component of sunscreen products (Middlemas et al., 2013). Organic and metal nanoparticles are also used as additives and functional compounds in food and food additives (Bouwmeester et al., 2014).

Airborne particulate toxicity can be studied in several ways. Examples are animal or human models *in vivo*, cellular cultures *in vitro*, and indirectly by using epidemiology (Pettibone et al., 2008; Wierzbicka et al., 2014). There are several advantages to the development of *in vitro* toxicological models. They are less

expensive and logistically simpler than many *in vivo* methods and chamber studies, as well as less ethically demanding compared to *in vivo* experimentation.

A challenge for *in vitro* toxicological research regarding airborne particulates is that of relevance. Much of the *in vitro* toxicological work performed has been carried out in an aqueous environment, that is, cells are grown on a substrate with a surrounding medium of nutrient, buffer and antibacterial agents. A development that addresses the relevance of *in vitro* toxicological research regarding airborne particles, is that of air-liquid interface research and methodology. This branch of *in vitro* toxicology focuses on delivering airborne particles directly onto cell cultures relevant for the respiratory system (Jeannet et al., 2014; Lenz et al., 2009; Savi et al., 2008). Since the particles are then deposited directly onto the cell lining fluid or mucosa, potential problems of administering them in solution can be overcome. Some such problems are aggregation, change of particle morphology and dose quantification in solution (Teeguarden et al., 2007). In order to create general models of the properties that are toxic and the ones that are not, it is, however, not enough to assert that some methodological issues have been overcome. For example, how particles interact with biomolecules and proteins at the air-liquid interface itself needs to be further studied so that the fate of the particles may be accurately predicted.

Traditionally, the preferred way of quantifying the number of particulates in an aerosol has been by gravimetric measurements, such as impaction or filter collection. In both Dockery et al. (1993) and Wilkins (1954), death rates were related to PM. In legislation and environmental monitoring, quantifying the mass PM content in an aerosol is still the most common measurement. It has been hypothesized, and to some degree shown, that PM does not tell the whole story. Studies have shown that health effects are also related to the total particle number concentration of an aerosol: The concentration of ultrafine particles (UFP) (particles below 100 nm in size) was able to predict the effects on peak expiratory flow and other respiratory effects in asthmatic adults (Peters et al., 1997). Human inhalation studies with carbon UFP have also reported a reversible effect on lung function in non-asthmatic subjects (Pietropaoli et al., 2004), and an effect on heart rate variability (Hagerman et al., 2014). The legislation on asbestos exposure is quantified in number of fibers rather than mass, reflecting asbestos great toxic potential. Research has also shown that for some types of particles, the surface area of the particulates better describes the toxic effect observed than either number or mass (Donaldson et al., 2008; Waters et al., 2009).

Now that the stage is set, the author hopes that you, dear reader, realize the urgency as well as difficulty of studying the effects of aerosol particles on our health. Challenges include relevance, characterization, dosimetry and measurement of the toxicological response. This thesis addresses all these challenges, as presented in the upcoming section.

## Aims of the thesis – The GCA

The overall aim of the research presented in this thesis was to develop and validate a holistic system for research pertaining to the toxicological and biological effects of aerosol particles, as well as their physical properties.

The system has a continuous, high-output aerosol particle source, with an option for variable shape, size and material. The aerosol particle output is highly characterized, so that the aerosol content can be described in terms of number, mass and surface area so that valid comparisons can be made with other studies. The system uses an air-liquid interface exposure chamber with a high deposition efficiency, and an electrostatic precipitator to collect samples for scanning electron microscopy (SEM), transmission electron microscopy (TEM) and biological and toxicological testing.

This Generation, Characterization and Air-liquid interface toxicological chamber system is denoted as GCA.

### **Aim 1 – Validation of nanostructured aerosol particle output characteristics of the GCA system**

Aim 1 was to validate the output and characteristics of the aerosol particle source of the GCA system according to three sub-aims:

1. To determine if the aerosol particle source provides a continuous and stable output flow of nanostructured aggregates, composed of primary particles, or near spherical sintered structures in the sub-micron size range.
2. To determine if a proposed power law adequately describes the size dependent mass of the aggregates. To also present and discuss the uncertainties related to the power law's determination.
3. To determine if the output of the source is adequately expressed in terms of number, mass or surface area for valid comparability with other studies.

### **Aim 2 – Validation of the GCA dose and cellular response**

Aim 2 validate if the GCA, with regards to cellular dose and response, is able to:

1. Ensure a high degree of particle deposition on the cellular cultures.
2. Produce a wide range of doses in terms of number, mass or surface area, comparable to those of other studies.

3. Detect significant effects on viability and cytokine expression by exposing primary and carcinoma cells to the achieved doses.

### **Aim 3 – Characterize particle interactions with proteins and biomolecules**

Aim 3 pertains to understanding how particles interact with proteins and biomolecules when administered on an air-liquid interface. The aim is divided into the following three research questions:

1. What is the first interaction that occurs when particles come into contact with a solution of protein and biomolecules?
2. Do the particles aggregate or form discrete complexes in solution with protein and biomolecules?
3. Does a protein corona form around the particles in solution?

# Theory and methods

## Toxicology in the laboratory

In short, the study of toxicology can be summarized in the famous quote by Paracelsus:

“Alle Dinge sind Gift und nichts ist ohne Gift. Allein die Dosis macht, daß ein Ding kein Gift ist.”<sup>1</sup>

The quote in essence states that it is the dose that makes the poison, not solely the compound.

### **In vitro**

In principle, in vitro experiments are performed on living cells or tissues outside the organism under known and controlled conditions. The principle can be traced back to the middle of the 20<sup>th</sup> century when Russell (1957) introduced the concepts of reduction, refinement and replacement. Since then, several major congresses have been held and major scientific funding provided. Today there are several in vitro based toxicological assays available from major retailers. These include cellular viability and cytotoxic potential. There is to date an enormous amount of scientific literature regarding in vitro particulate toxicity. The principal method of in vitro toxicological experimentation has been the exposure of cell cultures to solutions of toxicants.

### **In vitro – air-liquid interface style**

What the air-liquid interface exposure systems and techniques share in common is that the cell cultures, tissues or solutions are directly exposed to an aerosol. This is

---

<sup>1</sup> “Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy.”



in contrast to particulates that are sampled on filters, impingers or other collection devices and subsequently dispersed in solution for toxicological testing. The hypothesis spurring the air-liquid interface methodology and research is that toxicological testing of aerosol particles in a solution environment may be unrealistic, and in the end may produce erroneous results. To date, many in-house devices have been developed and described in the literature, as well as commercially available systems for air-liquid interface research. There are different variants with respect to how the particles are actively or passively administered onto the liquid, mucosa or cell culture surface (Figure 1).

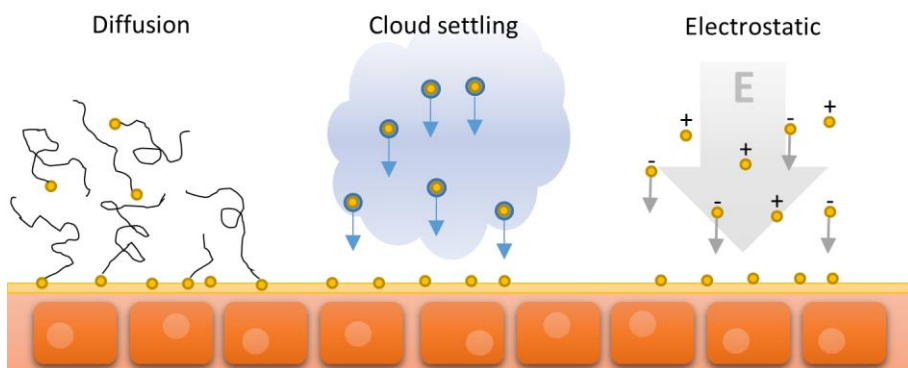


Figure 1. Passive (diffusion) and active (cloud settling and electrostatic) deposition mechanisms for an air-liquid interface exposure system.

Based on a brief literature search, one of the earlier air-liquid interface devices for toxicological research was the rocking-cradle, as described in Bombick et al. (1997). The rocking-cradle shifts the culture media from side to side in the culture flask, while exposing the system to an aerosol of interest. The shifting of the media allows for a portion of the cell culture to be directly exposed to the aerosol. The rocking cradle is a passive device in the sense that no additional forces or effects are imposed on the particles to force them to the cell culture surface; only diffusion, sedimentation and settling are in effect. The Vitrocell exposure chamber is also a passive system that primarily uses diffusion for the deposition of aerosol particles (Persoz et al., 2011), (Figure 1). An exposure system with active delivery of particulates is described in Lenz et al. (2009), the Air-Liquid Interface Cell Exposure system (ALICE). The device uses a cloud settling technique: water condenses on the particulates and they become sediment on the cell culture surface (Figure 1). Devices that utilize electrostatic forces to ensure a high degree of particle deposition are CULTEX, first described by Aufderheide and Mohr (2000), and the Nano Chamber In Vitro Toxicity (NACIVT) system (Jeannet et al., 2014; Savi et al., 2008) (Figure 1). The NACIVT system combines an incubation environment, unipolar charging of aerosol particles and static or alternating electrical deposition fields in 24 individual compartments.

## Is air-liquid interface toxicology more sensitive?

Studies have shown a difference between the suspension and the GCA methods. A brief literature comparison is presented in Table 1.

Table 1. Brief comparison of studies showing a discordance between suspension-based and GCA toxicological methods. The overview indicates whether the GCA-based results suggest a lower or higher sensitivity compared to results based on suspension.

	Effect shown by GCA compared to suspension	
	Less sensitive	More sensitive
Holder et al. (2008)		x
Fröhlich et al. (2013)		x
Lenz et al. (2013)		x
Panas et al. (2014)	x	

The comparison suggests that the GCA methodology may well be more sensitive than the corresponding suspension toxicology. Potential explanations include a more direct route of exposure to the cellular systems and a difference in cellular dose. It has also been suggested that the rate of dose delivery in vivo may affect the toxicological outcome (Baisch et al., 2014). This hypothesis helps to explain the differences between suspension and air-liquid interface toxicological research.

## Particles in solution

Particles collected or dispersed in solution may either aggregate, deaggregate, become sediment, coalesce, dissolve or by diffusion adsorb on surfaces (Figure 2).

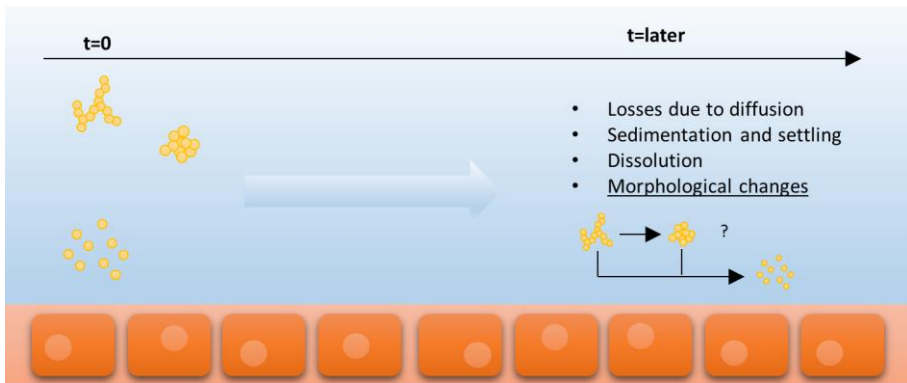


Figure 2. The fate of a particle in solution. Depending on material and surface properties, it may aggregate, deaggregate, coalesce or dissolve.

A practical implication of this change is that the sedimentation and transport vary for particles in suspensions of different sizes. This effect has been recognized in the scientific literature and there are corrections and methods for calculating this flux, as dependent on particle properties (Teeguarden et al., 2007).

There are an array of techniques available to determine hydrodynamic size; below are two techniques that were used **Paper II**.

### Dynamic light scattering

Dynamic light scattering (DLS) is a widely used technique to determine particle hydrodynamic diameter in solution. A laser is directed through a sample of the solution and a detector at a known angle collects scattered light. The light pattern recorded at time zero deteriorates over time, that is, as the particles in the solution diffuse the light pattern changes. How fast the pattern changes is then related to the hydrodynamic size of the particles in the solution.

The inversion by which the hydrodynamic size is determined is quite complex and is not covered in this thesis.

### Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) also uses laser for its measurements. Compared to DLS, however, NTA is much easier to comprehend from a mathematical perspective. A detector is placed perpendicular to the laser, collecting scattered light. Each particle is tracked by its scattered light, allowing for a calculation of diffusivity and hydrodynamic size for individual particles.

## Nanoparticle protein corona

The term “protein corona” refers to a phenomenon when proteins bind to the surface of particles and form a covering surface. Even though there is no strict definition, it is most often used for how proteins adsorb on nanoparticles and nanostructured materials. The term had a large impact on the scientific community with the Cedervall et al. (2007) and Lundqvist et al. (2008) papers. For reference, Cedervall et al. (2007) concluded that for a 70 nm nanoparticle, approximately 620 human serum albumin proteins form a complete corona, and for a 200 nm nanoparticle, about 4650 proteins. An albumin protein is approximately 6-7 nm in hydrodynamic diameter, as visualized in Figure 3 in comparison to a nanoparticle.

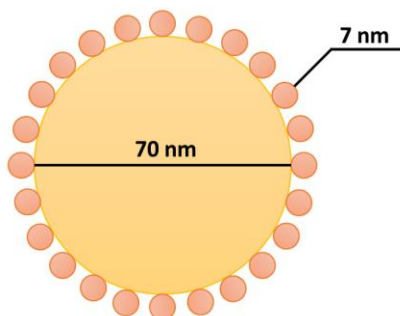


Figure 3. Comparison of the hydrodynamic diameters of a 70 nm nanoparticle and a 7 nm protein (the size of albumin).

It is worth noting that previous to Cedervall et al. (2007) and Lundqvist et al. (2008), there were studies that focused on how particles interacted with proteins. For example in Müller et al. (1997), the binding of the plasma protein was studied on fluorescent and nonfluorescent 100 nm particles. Plasma protein adsorption has also been studied as being dependent on nanoparticle hydrophobicity in Gessner et al. (2000).

### **Gel electrophoresis – SDS PAGE**

In order to study how particles interact with proteins in solution, sodium dodecylsulphate polyacrylamide gel electroforesis (SDS PAGE) can be performed. In short, a gel is formed at which the suspension of interest is introduced. An electric field forces the proteins of the solution to migrate through the gel. The methodology can be perceived as a mass to intensity spectrometer with low time resolution.

## Particle sizing in gas

Particles of special interest for this thesis are aggregates and agglomerates. These are clusters of smaller primary particles. Aggregates are partly fused and agglomerates are lightly bound together. The nomenclature used in **Paper III** will be used in this thesis: an aggregate consists of smaller primaries tightly fused with a bridge; agglomerates are primaries that are weakly bound by electrical van der Waals forces. Determining and reporting the size of spherical objects is easy. Significantly more difficult is reporting the size of an irregular object such as an aggregate or agglomerate, or simply determining its most descriptive characteristic, Figure 4A.

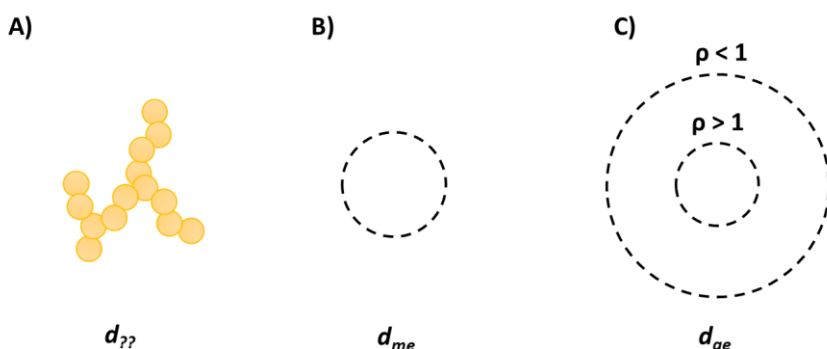


Figure 4. A) An aggregate of smaller primary particles fused together. The particle does not have a distinct geometrical size by which it can be described. B) By determining the particle's motion in an electrical field, an equivalent diameter can be reported. This diameter is closely related to the diffusivity of the particle and is called the electrical mobility diameter  $d_{me}$ . C) The aerodynamic particle size,  $d_{ae}$ , can be determined by studying the particle's inertial properties, which may result in a greater or smaller value than that of the mobility diameter depending on the density of the particle.

One way of circumventing this difficulty is to use equivalent diameters. For submicron aerosol particles, a common diameter is the equivalent calculated from electrical mobility ( $d_{me}$ ). The  $d_{me}$  can be classified using a differential mobility analyzer (DMA). Particles are streamed through a space between two electrodes, carried by a gas stream. Particles of a specific mobility,  $Z$ , will pass through the DMA and by using an equation derived from Stokes' law:

$$Z = \frac{n \cdot e \cdot C_c}{3 \cdot \pi \cdot \eta \cdot d_{me}} \quad (1)$$

The  $Z$  is also dependent on the number,  $n$ , of charges,  $e$ , the Cunningham factor,  $C_c$  and viscosity,  $\eta$ , of the gas. A DMA in combination with a condensation particle

counter (CPC) and a data inversion technique comprise a scanning mobility particle spectrometer (SMPS) (Wang & Flagan, 1990).

Particles can also be characterized by their inertial properties, for example in an impactor or an aerodynamic particle sizer (APS). An equivalent diameter,  $d_{ae}$ , is calculated assuming a density of  $1\text{g/cm}^3$ . This is because the density of the particle matter for the inertial properties of the  $d_{ae}$  may also be both greater and smaller than the  $d_{me}$ , Figure 4C.

## Particle mass

This section presents the methods for determining aerosol particle mass concentration, and for determining mass mobility relations online.

### Aerosol particle mass analyzers

By determining the mass and density of aggregates, a determined size number distribution can be transformed to size mass. Important clues as to the properties of the aerosol can also be inferred.

The size dependent mass of an aerosol of aggregates can be determined by using a DMA and aerosol particle mass analyzer (APM) in combination with a particle counter. The basic operating principle for an APM is that an aerosol is introduced in a space between two rotating cylindrical electrodes. The voltage,  $V$ , between the electrodes is set along with their angular velocity,  $\omega$ :

$$\frac{m}{q} = \frac{V}{r_c^2 \cdot \omega^2 \cdot \ln\left(\frac{r_2}{r_1}\right)} \quad (2)$$

By assuming a single charge,  $q$ , the mass,  $m$ , may be calculated;  $r_c$ ,  $r_1$  and  $r_2$  are parameters relating to the dimensions of the rotating cylinders, further described for Kanomax 3600 in Ehara et al. (1996). When both the mobility diameter and particle mass is known, the effective density,  $\rho_{eff}$ , and dynamic shape factor,  $\chi$ , can be calculated as further described in **Paper I & III**.

### The mass mobility relationship for aggregated particles

The relation between mass and mobility for an aerosol particle can provide clues as to its properties, as well as permitting the transformation of number to mass

distributions. For aggregates, one such property is the mass mobility exponent,  $D_{mm}$ , derived from the relation:

$$m_{agg(d_{me})} = k \cdot d_{me}^{D_{mm}} \quad (3)$$

where  $m_{agg}$  is the size dependent aggregate mass and  $k$  is called the K-factor. The value of  $D_{mm}$  can give insight into the formation process of the aggregate. Possible processes include reaction limited, diffusion limited and ballistic types.

The mass mobility exponent can be traced back to the concept of fractal dimension,  $D$ , as it relates to the number of primary particles,  $N$ , and the radius of gyration,  $r_g$ :

$$N \propto r_g^D \quad (4)$$

The radius of gyration is a concept closely related to the size of an aggregate. In short, it can be described as relating the distance of the primary particles to the aggregate center of mass. The radius of gyration does not scale in linearly to the mobility diameter, as is summarized in Sorensen (2011). This means that the fractal dimension may not be directly comparable to the mass mobility exponent.

## Determination of aerosol mass concentration

Historically, aerosol mass concentrations have been determined gravimetrically using, for example, filter collection or impactors, which are still used in many applications. Dockery et al. (1993) used sampling of the total suspended particulate matter to determine exposure in the six city populations. Today there are online instruments such as the tapered element oscillating microbalance (TEOM, Thermo Sci.), an instrument used in the **Paper IV** study, as well as optically based instruments.

By transforming number size distributions, derived from SMPS measurements (for example) to mass distributions is also a way of determining aerosol mass concentration. This requires the mass mobility relation to be known for the aerosol particles and is performed in the studies presented in **Papers I, III and IV** of this thesis. A transformation can also be performed to obtain the surface area distribution if the specific surface area is known.

## Morphological properties and surface area

Intuitively the surface area of an object is easy enough to understand. And for perfect geometrical objects, such as spheres or cubes, surface area calculations and

interpretations are relatively simple. Considering a more complex structure such as a metal or soot aggregate in the  $<100$  nm size range, this calculation is, however, not as simple as that of a perfect sphere.

Aggregates and agglomerates are composed of smaller primary particles. By determining the primary particle distribution of aggregates, the specific surface area (SSA) for the particles can be calculated, and when combined with the size mass distribution, the size surface area distribution can be determined. A typical way of determining the primary particle size is to use image analysis software to take manual measurements of TEM samples (Figure 5).

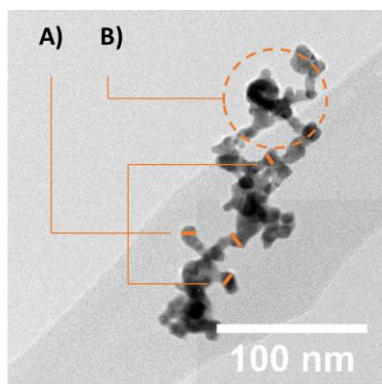


Figure 5. A gold aggregate as visualized by transmission electron microscopy for primary particle analysis. A) Typical measurements taken from primary particles of the gold aggregate. B) Structures on the aggregate not explained by the primary particle size.

The SSA can then be calculated using the Sauter primary particle diameter, a derivative of the whole primary particle distribution, as detailed in **Paper III**.

This approach typically does not take into account structures that are not explained by a primary particle size; these structures can be those illustrated in Figure 5B. Another property of aerosol aggregates that affects their relation between mass and surface area is bridging. This is when there is no clear distinction between primary particles (i.e., they can be said to be fused together).





# Results and discussion

## Aim 1 – Variable, characterized and stable output of nanostructured aerosol particles by the GCA system

Overall, the three generators tested were suitable with regards to the characteristics and output of nanostructured particles.

### Particle output

The commercial spark discharge generator (SDG<sub>P</sub>, Palas model 1000), the high temperature evaporation furnace (HT), and the spark discharge generator developed at Solid State Physics, Lunds University (SDG<sub>C</sub>) were evaluated with regards to particle output. The SDG<sub>P</sub> and HT generators were evaluated using gold as the base material. The number size distributions for the two SDG<sub>P</sub> settings investigated were below 100 nm while the HT settings had some part of their distribution over 100 nm. The gold aggregates generated were composed of smaller primary particles, <10 nm. The sintered structures were near spherical and had a number size distribution below 100 nm (**Papers I and III**).

The SDG<sub>C</sub> generator output was found to be stable and continuous over 1 hour for Cu, Pd and Ag aerosol particles, that is, the size distribution and particle number output did not vary to a large extent over this period (Figure 6).

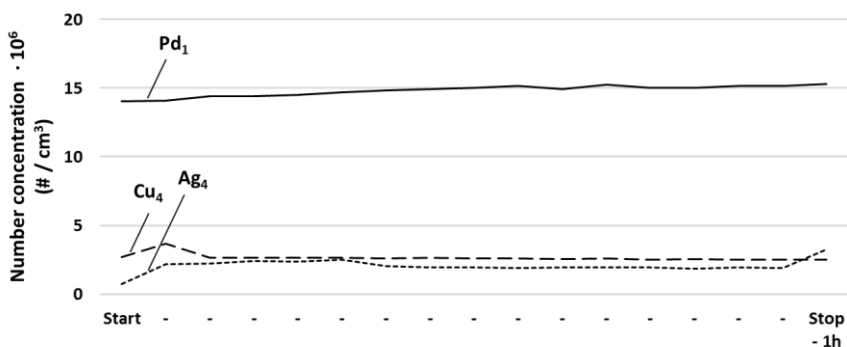


Figure 6. The output of the SDG<sub>C</sub> spark discharge generator with respect to total particle number concentration, reported from SMPS data. Aerosol particles of Pd, Cu or Ag aggregates were generated with a stable output during 1 hour periods, corresponding to the length of the exposures in the NACIVT of **Paper IV**. The Cu, Pd and Ag aerosol is indexed and full data can be found for the aerosol particles in **Paper IV**.

Aggregates generated from Cu and Pd were by visual analysis clearly composed of smaller primary particles, <10 nm, forming larger aggregates. The sintered aggregates were also near spherical shapes. The number size distribution of the Cu and Pd aggregates were lognormal distributed and had approximately 50 % of the distribution above 100 nm. The aerosol of sintered near spherical Cu and Pd had a number size distribution of primarily <100 nm (**Paper IV**). The SDG<sub>C</sub> was also evaluated with gold as the electrode material. The aggregates generated were also composed of smaller primary particles, and the principal part of the number size distribution was below 100 nm (**Paper III**).

The significance of 100 nm is that it is the upper limit recommended in the European Commission's definition of nanomaterial (European-Commission, 2011). Since the primary particles are below 100 nm for all three generators and gold was the material, the particles can accurately be called nanostructured because they have portions of their aggregate size distributions in the <100 nm range.

### Particle size dependent mass

The size dependent masses of the aggregates generated by the SDG<sub>P</sub>, HT and SDG<sub>C</sub> were well described by the power law in eq. 3. There were no significant differences in  $D_{mm}$  for the Au aggregates generated by the SDG<sub>P</sub>, HT or SDG<sub>C</sub>, or for the Cu and Pd aggregates generated by the SDG<sub>C</sub> (**Papers III and IV**) (Figure 7A).

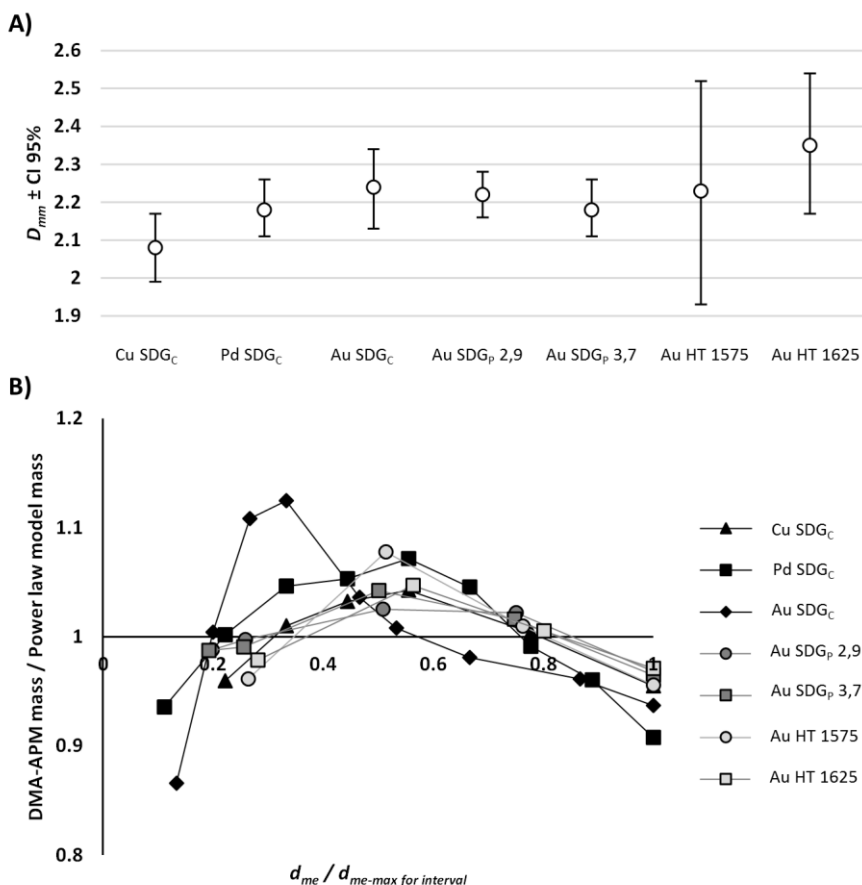


Figure 7. A) The mass mobility exponent of aggregates of Cu and Pd generated by SDG<sub>C</sub>, Au generated by SDG<sub>C</sub> and SDG<sub>P</sub>, and Au generated by HT. The error bars represent the 95 % confidence interval of  $D_{mm}$  as determined from regression analysis. B) Analysis of how the measured aggregate mass, by DMA-APM relate to the mass calculated by its corresponding power law model, determined by regression analysis. The x-axis is a normalized mobility diameter (i.e., each mobility diameter was normalized to the maximum diameter in its measurement interval). This was performed to make comparison possible even when the DMA-APM measurement intervals differed between materials, methods and settings. The results show that the aggregate mass calculated from the fitted power law model, determined from regression analysis, systematically differs from the actual measurements in a non-linear fashion.

In addition to the calculation of  $D_{mm}$  and its uncertainty, an analysis was carried out of how the aggregate mass varied in relation to its counterpart, which was calculated by the power law (Figure 7B). In order to compare different DMA-APM aggregate mass-to-model ratios, the x-axis in Figure 7B was normalized. Each  $d_{me}$  was normalized to the maximum  $d_{me-max}$  in the measurement interval. For example, if the aggregate mass is determined for 20, 40 and 60 nm, the normalized value for the  $d_{me}$  would be 0.3, 0.6 and 1, respectively.

The results of this analysis show that there is a systematic non-linear discrepancy between the actual aggregate masses determined by the DMA-APM and their corresponding power-law calculated values. This indicates that the uncertainty determined for  $D_{mm}$  in **Papers III** and **IV** and summarized in Figure 7 most likely is explained by this non-linear discrepancy. A potential explanation for this discrepancy is that the data are not corrected for double charge. The effect of double charge on particle mass, as determined by DMA-APM, is presented and discussed in Rissler et al. (2013). Another explanation could be that the aggregate properties, such as bridging and/or primary particle size, vary in a non-linear size dependent manner.

## Particle output in terms of number, mass and surface area

The mass size distribution of aggregates could be calculated for the generation methods being validated for the GCA by applying the power law obtained from the mass mobility relationship described in the previous section. Pd aggregates are illustrated in Figure 8A.

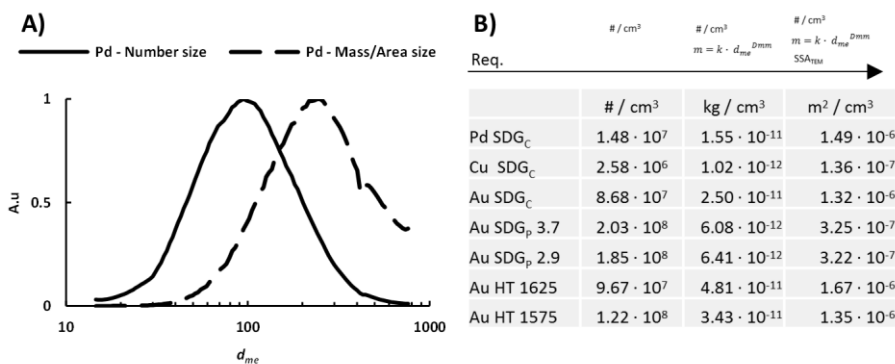


Figure 8. A) Number, mass and surface area distributions of Pd aggregates generated with the SDG<sub>C</sub> method. B) Graphic of all validated aerosol particle generators with the number, mass and surface area output. The top of the table shows the requirements for calculation. The calculation of surface area was performed according to the Bau-Rissler Method of **Paper III**.

By using the SSA of the aggregates, the surface area distribution could also be determined. The sum of the distributions each represents the total number, mass or surface area content. This methodology was applicable to all aerosol particle generators being validated, as shown in Figure 8B. The method of calculation was that of the Bau-Rissler method described in **Paper III**.

## Aim 2 – Validated the GCA dose and cellular response

The cellular exposure chamber evaluated for the GCA, the NACIVT, had a high degree of particle deposition. The dose could be calculated in terms of number, mass and surface area and cellular responses could be observed.

### Deposition efficiency and dose

In **Paper IV** the deposition efficiency was evaluated using sintered Ag aggregates. This was performed by depositing sintered Ag particles onto silicon wafers in the NACIVT. The deposition efficiency was calculated to 36 % with a standard deviation of 17 %. This is comparable to the values from Savi et al. (2008), which were used in **Paper I** for dose calculations and that also determined in Jeannet et al. (2014).

**Paper I** used output data from the SDG<sub>P</sub> and HT generated aerosols of Au aggregates along with the deposition efficiency data reported in Savi et al. (2008). Based on these two sets of data, it was concluded that a dose of 5-35  $\mu\text{g} / \text{cm}^2_{\text{cell}}$  could be achieved in 1 hour of deposition in the NACIVT, the corresponding surface area dose being 1-7  $\text{cm}^2_{\text{particle}} / \text{cm}^2_{\text{cell}}$ . The dose was calculated for three methodologies and the results for all three were in the same size range.

For the SDG<sub>C</sub>, the doses of Cu, Pd and Ag aggregates and sintered near spheres were calculated using the deposition efficiency of 36 % determined in the **Paper IV** study. First the number size distributions were transformed into mass and surface area distributions, detailed in **Paper III**. The dose was then calculated according to the methodology described in **Paper IV**. The doses were in the range of 0.4 - 46  $\mu\text{g} / \text{cm}^2_{\text{cell}}$  per hour of deposition, depending on particle material and dilution.

The dose range produced for Au, Cu, Pd and Ag were comparable to that of other studies performed using an air-liquid interface toxicological approach. This is discussed in detail in **Paper IV**.

### Determination of deposition efficiency can be problematic

For aggregates of Cu and Pd, deposited in high doses on silicon wafers, patterns consistent with electric field alignment and condensation were observed. There were significant overlaps and clustering of aggregates on the wafers. The sintered structures also formed clustered patterns, **Paper IV**.

These phenomena need to be taken into consideration when determining the deposition efficiency. It is clear that for low dose levels, these phenomena were less pronounced compared to high dose levels. Consequently, it is strongly

recommended that the analysis of deposition efficiency should only be performed based on low dose levels.

### **Both response and dose response were observed**

By generating nanostructured aerosol particles with the SDG<sub>C</sub>, both primary small airway epithelial cells (SAEC) and carcinoma cell line (A549) were used for exposure in the NACIVT. Aerosol particles of Cu, Pd and Ag were generated continuously during 1 hour exposures; different levels of exposures were set using a dilutor. The exposures were monitored as described in **Paper IV** using both SMPS and TEOM.

The effects of the nanostructured particles on the cell cultures were analyzed in three tiers, explained in further detail in **Paper IV**.

1. The first tier considered only if the cells had been exposed to aerosol particles for a 1-hour duration in the NACIVT. No regard was taken to analyzing particle material, morphology or dose.
2. The second tier also considered particle material but not morphology or dose.
3. The final tier considered all aspects of the particles, including dose and morphology. This made the analysis of dose response possible.

In the first tier, significant effects were observed for SAEC and all endpoints except MCP expression; for A549, no significant effects were observed. When differentiating with regards to particle material, significant effects were seen in A549, WST-1, for Cu and Pd. For SAEC and WST-1, all materials had a significant effect. For the cytokine analysis, Pd only gave significant result in il-6, Cu for il-8 and TNF- $\alpha$ , Ag for il-6, MCP and TNF- $\alpha$ . When exposed to Pd, the SAEC expression of TNF- $\alpha$  was below the level of detection.

The dose was expressed as number, mass and surface area with regards to dose response. This was done in order to investigate if any of the metrics could coherently explain the toxicity. When the dose was expressed in terms of number, a significant dose response ( $p < 0.05$ ) was observed for SAEC, il-6, exposed to Pd. For SAEC, WST-1, exposed to Cu, when the dose was expressed in terms of mass, a significant dose response was produced. For TNF- $\alpha$  expression as a result of Cu and Ag exposures significant dose response was observed with dose expressed in terms of number, and also for Ag with dose expressed as mass. No other significant dose responses were observed. This finding is further discussed in Aim 3, in relation to the findings in **Paper II**.

## Comparability with real life exposure

In Gangwal et al. (2011) a dose of Ag particles in the size range of 5-100 nm based on 24 hour exposure to  $1 \text{ mg/m}^3$  was estimated to  $0.06\text{-}0.15 \text{ }\mu\text{g/cm}^2$ . The working lifetime dose, after 45 years of exposure is approximately 10-100 times higher. The dose of Ag in **Paper IV** is at its highest, approximately  $2\text{-}3 \text{ }\mu\text{g/cm}^2_{\text{cell}}$ , corresponding to a near lifetime dose. Since Cu and Pd are reasonably similar in density to Ag, the 24-hour and lifetime doses can be approximated to lie within a factor 2 of that calculated for Ag. This would mean that the highest Pd dose in **Paper IV** corresponds to more than 10 times a lifetime dose, for similar exposure to Ag in Gangwal et al. (2011).

Demokritou et al. (2013) reported a dose, in a murine lung in vivo model of  $0.57 \text{ }\mu\text{g/cm}^2$  for CeO at a exposure of  $2.7 \text{ mg/m}^3$  for a duration of 8 hours, count median diameter (CMD) in the 100 nm range. Even though this thesis is not concerned with CeO, it serves as an important contribution to our frame of reference (i.e., what is toxicologically reasonable and relevant).

Finally, in reference to Paur et al. (2011), a lifetime dose of  $420 \text{ }\mu\text{g/cm}^2$  is calculated for exposure to  $5 \text{ mg/m}^3$  during a full working lifetime. The aerosol particles were in the size range 10–100 nm and the value is corrected for long-term clearance in the lung. Urban environmental exposure is approximated in a similar fashion to a lifetime particle dose of approximately  $6.6 \text{ }\mu\text{g/cm}^2$ , also corrected for some clearance.

## Aim 3 – Particle interactions with proteins and biomolecules were characterized

Aerosol particles of Au, generated by HT as described in **Paper I** and **III**, were used to study the interactions between particles and the air liquid interface of biological solutions.

### Film formation at the air-liquid interface

A red film was observed at the air-liquid interface when spherical gold nanoparticles were deposited onto solutions of buffered bovine serum albumin (BSA), serum and lung fluid. When depositing similar amounts of gold aggregates of the same mobility size, no red film was observed. The film was deemed stable over a time frame of at least hours and did not dissolve on its own. When dissolved, by stirring and/or repeated pipetting, complexes of proteins and particles formed in the solution rather than larger aggregates being formed **Paper II**. This may also have



implications for the deposition of particles onto the thin liquid layer covering the cells in the deposition chamber.

This thesis proposes, on the basis of the previously described findings of **Papers II** and **IV**, that the particle film formation at the air-liquid interface can function as a protective barrier during some conditions. This can explain why the significant dose-response relations in **Paper IV** could only be significantly asserted in a few cases.

A plausible hypothesis is that the air-liquid interface is saturated with particulates, and once saturated, an increase in dose has little or no effect on the response of the cell cultures. The lack of visible film formation when depositing aggregates is hypothesized to be the result of their difference in light scattering properties compared to spheres.

It is clear from the results of this thesis research that the film formation needs to be investigated. Likely research questions concern the conditions under which this film forms and its long-term stability, as well as if it can form with soluble particles. Further study of the deposition in lung fluid could offer clues as to the particle's fate with regards to its properties. The methodology described in **Paper III** would be well suited for such studies.

### **Particle hydrodynamic size in biological solutions**

When sintered Au nanoparticles and aggregates, 60 nm in diameter, are deposited onto biological solutions a film is formed, as discussed in the previous section. The film could be easily dispersed after which complexes of particles and proteins formed, as summarized from **Paper II** in Figure 9.

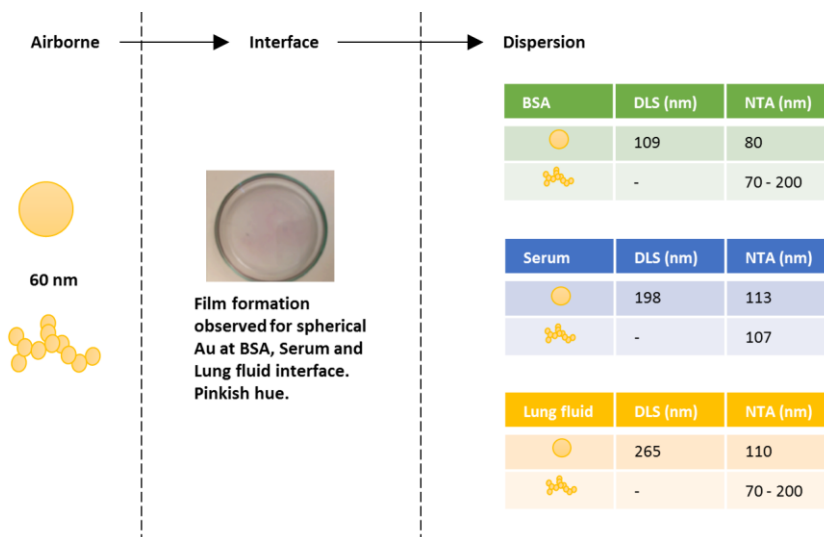


Figure 9. Aggregates generated by high temperature condensation and sintered near spherical Au were deposited onto biological solutions. For the spherical Au, a pink film formed at the air-liquid interface of BSA, serum and lung fluid solutions. This film could not be observed for the deposited aggregates. The particles formed stable complexes with proteins when the film was dissolved. Both dynamic light scattering (DLS) and the nanoparticle tracking analysis (NTA) techniques were employed. The image of film formation is from a Master's thesis project on the topic of this film formation (Adner, 2015).

Aggregates deposited onto a biological solution did not produce a pink film. In **Paper II** there is a discussion about how this may be due to the smaller primary particles in aggregates being the ones that determine the light scattering properties. The pink hue of the film can be explained by the plasmon effect of spherical Au in the size range of 60 nm.

When spheres and aggregates are deposited onto a homocysteine solution, no film was observed and large flakes of aggregated particles form instead of stable complexes (**Paper II**).

### A protein corona was formed

The results from **Paper II** show that distinct protein corona patterns can be determined for Au particles deposited onto diluted porcine serum, using the methods described in the *Theory and Methods* chapter. The results show that the corona is essentially similar for spherical and aggregate 60 nm particles. The corona is also discussed in **Paper II** to be of a different character than Au, similar in size but with surface modifications.

The protein corona of particles has long been hypothesized to be important for the particles' biological fate (Lundqvist et al., 2008). In Salvati et al. (2013), it was also shown that the corona can have a direct effect on the intended effect of particles on cells.

Thus, a reasonable hypothesis is that Au particles, or particles in general, that have similar composition and size may affect cells differently based on their coronas. According to the findings in **Paper II**, it is possible that a GCA can produce results that are different than traditional toxicology.

# Outlook

## Considering the logistics

The methodology for the characterization of particles in solution in **Paper II** was not realized in **Paper IV**. This is due in a large part to logistical reasons, the limiting factors being manpower and space. For example, when teams from different disciplines team up to perform experiments, it is not unusual that the experimental resources are scattered around the campus. This needs to be considered when planning experiments with a GCA system.

For this type of toxicological, biochemical and physical research to achieve its full potential, a step beyond the multidisciplinary approach has to be taken. In essence, a physicist can no longer afford to be just a physicist, nor can a toxicologist or biochemist afford to be narrow in ability or knowledge.

## Toxicology and the future

The OECD and the European Union's Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) have several standardized, in vitro tests for skin irritation, fertility and cancerogenity. However, the gap between in vitro and in vivo is large and currently there is no bridge that completely closes it. Through Directive 2010/63/EU, the EU has also legislated standards regarding the care and protection of animals used for experimentation (European-Commission, 2010). The principles of the legislation are to reduce, refine and replace the use of animal models in research as first introduced by Russell (1957). This means that the development and application of relevant in vitro models are of great importance for future toxicological research.

## Improving the GCA

A second iteration of the GCA system design could be subject to interesting changes and possibilities. For example, an online surface area monitor could be used in

parallel with the SMPS for constant monitoring of the aerosol particle generation to the NACIVT. An important aspect of the methodology is that of deposition efficiency. A second iteration would include a method for its reliable and practical determination, as is briefly discussed in **Paper IV**.

An alternative to TEOM measurements can also be considered. The rationale is that when TEOM has been subject to a change in pressure, it can take 15-30 min before reliable measurements can be taken. The filter can also overload quickly if the aerosol is not diluted before the TEOM inlet.

## The dose is very high . . .

When discussing in vitro toxicology, a common reservation is if the dose levels are far too high, or at all relevant.

It is clear that doses produced and administered during in vitro experimentation can be considered as high. Some of the dose levels produced in **Paper IV** and the related air-liquid interface toxicological studies may only be relevant for real life situations with extreme particulate exposures. In addition, these lifetime doses are administered during very short time periods, on the time scale of hours as compared to decades in real life. In many cases these high doses are rationalized by a lack of response to lower doses (i.e., in order to see some effect, studies are forced to use unrealistic doses of particulate material). The field of toxicological research, however, is not standing still, and the time of unrealistic doses may yet pass. Developments in genomic and proteomic research can potentially allow for the detection of subtle changes in cell cultures as a result of particulate exposure. Developments in cell cultures and tissue explants could also allow for more relevant and sensitive model systems.

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