SEVENTH FRAMEWORK PROGRAMME

THEME 4 - NMP – NANOSCIENCES, NANOTECHNOLOGIES, MATERIALS, AND NEW PRODUCTION TECHNOLOGIES (214281)

NANOMMUNE

COMPREHENSIVE ASSESSMENT OF HAZARDOUS EFFECTS OF ENGINEERED NANOMATERIALS ON THE IMMUNE SYSTEM

QUALITY HANDBOOK

STANDARD PROCEDURES FOR NANOPARTICLE TESTING

[WORK PACKAGES NO. 2,3,4,5,6]

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1 Preface

Engineered nanomaterials (ENs) present tremendous opportunities for industrial growth and development, and hold great promise for the enrichment of the lives of citizens, in medicine, electronics, and numerous other areas. However, there are considerable gaps in our knowledge concerning the potential hazardous effects of ENs on human health and the environment. The NANOMMUNE consortium is committed to filling these knowledge gaps through a comprehensive assessment of ENs, with particular focus on effects on the immune system. The immune system is designed to respond to pathogens and foreign particles, and a core concept underpinning the current project is that the recognition versus non-recognition of ENs by immune-competent cells will determine the distribution as well as the toxicological potential of these materials. Our international, multidisciplinary consortium focused on the procurement, synthesis and detailed physico-chemical characterization of representative categories of ENs, and the monitoring of potential hazardous effects using an array of in vitro and in vivo systems, as well as transcriptomic and oxidative lipidomic profiling strategies to determine specific nanotoxic profiles (signatures) of these materials. The final and integrative component of our research project is modeling and risk assessment of potential adverse effects of ENs on human health, and the dissemination of our findings. Through our comprehensive approach, which combines analytical procedures from many different disciplines and leading experts from several national institutes devoted to occupational and environmental safety, we aim to establish a panel of read-out systems for the prediction of the toxic potential of existing and emerging ENs, thus enabling a continuous and sustainable growth of the nanotechnologies. Overall, the results generated through this international program will contribute to the understanding and mitigation of possible adverse effects of nanomaterials.

Introduction, scientific/industry needs, problem addressed

Nanotechnologies are viewed as being the driving force behind a new industrial revolution which is expected to have profound socio-economic effects. Nanotechnologies comprise a disparate array of technologies that cut across many traditional scientific disciplines, including chemistry, material science, engineering, physics, biosciences, medicine, and environmental sciences. The only unifying feature is the nanoscale dimensions at which the material concerned is being manipulated. Nanoparticles have all three dimensions in the nanoscale, whereas nanotubes have two dimensions in this regime, and nanosurfaces have one dimension in this regime. It is important to note that nanomaterials can be on the same scale as elements of living cells, including proteins, lipids, nucleic acids, and organelles [1]. Therefore, one must focus particular attention on how ENs can interact with or influence biological systems, which may be desirable for certain medical applications, but may cause unanticipated hazardous effects upon occupational or environmental exposure to nanomaterials.

The properties of materials are different on a nanoscale for several reasons. First, ENs have, relatively, a larger surface area than the same mass of material produced in a larger form. This can make materials more chemically reactive, and affect their functional properties such as mechanical strength or electrical properties. Second, below 50 nm, the laws of classical physics give way to quantum effects, provoking optical, electrical, and magnetic behaviors different from those of the same material at a larger scale. However, the very same properties that make ENs so uniquely

useful, such as a high degree of chemical reactivity and the ability to cross biological barriers may also be associated with unforeseen adverse effects on health and the environment. Moreover, small size *per se* may contribute not only to optimized transport conditions within the body [2] to the failure of immune recognition and hence to adverse or unexpected effects of nanoparticles. Indeed, numerous physico-chemical attributes, including size, shape, surface area, surface chemistry, solubility, charge, porosity, etc have been suggested to be associated with the potential adverse effects of ENs. However, much more research is required to ascertain the relevance of a given physico-chemical parameter for EN-associated toxicity following human exposure.

Maynard et al. [3] have proposed that the pursuit of responsible and sustainable nanotechnologies can be tackled through a series of grand challenges to stimulate the global research community, including the development and validation of methods to evaluate the toxicity of ENs, and the development of risk assessment models for predicting the potential impact of ENs on human health and the environment. Indeed, despite the tremendous growth potential of the nanotechnologies, there is still a considerable lack of information on bioaccumulation, biotoxicity, and biodegradation of ENs in humans as well as in other species. However, previous epidemiological studies have documented a strong association between so-called ultrafine air pollution particles and respiratory and cardiovascular morbidity and mortality in humans. Some, but not all of these effects, may be related to indirect actions of particles on components of the immune system, for instance through modulation of inflammatory cytokine secretion. Indeed, as pointed out by Dobrovolskaia & McNeil [4], ENs can either stimulate or suppress immune responses; moreover, these authors suggest that one of the fundamental questions in the field concerns the mechanisms through which nanoparticles are recognized by the immune system.

Scope, objectives of the consortium

Engineered nanomaterials present tremendous opportunities for industrial growth and development, and hold great promise for the enrichment of the lives of citizens, in medicine, electronics, and numerous other areas. However, there are considerable gaps in our knowledge concerning the potential hazardous effects of ENs on human health and the environment. The NANOMMUNE consortium (see diagram below) is committed to filling these knowledge gaps through a comprehensive assessment of ENs, with particular focus on effects on the immune system, our primary defense system against foreign invasion.

One challenge in evaluating risk associated with the production and application of nanomaterials is the diversity and complexity of the types of materials available, and the many different routes of entry and possible sites of interaction with biological systems. Our interdisciplinary project focused on the manufacturing and detailed physico-chemical characterization of several representative classes of nanomaterials, and the monitoring of deleterious effects of these nanomaterials on the immune system, using an array of *in vitro* and *in vivo* methodologies, as well as state-of-the-art *in silico* approaches for the assessment of genomic and oxidative lipidomic “nanotoxicity-signatures”. Our studies also included several examples of commercial ENs that are currently on the market. Moreover, we also modified specific features of various classes of ENs, in order to mitigate toxic responses to these materials.

The immune system, present throughout the body, and on constant surveillance, has the capacity to respond to invasion by pathogens and foreign particles. The core concept underpinning the

current project is that the recognition versus non-recognition of ENs by immune-competent cells will determine the distribution as well as the toxic potential of these novel materials. Moreover, we assessed whether ENs interfere with key functions of the immune system *in vitro* and *in vivo*, such as macrophage engulfment of apoptotic debris and antigen-presentation or exosome production by dendritic cells to lymphocytes. Through our comprehensive approach, which combines analytical procedures from many different disciplines, we established an array of read-out systems for the determination of toxicity not only of currently existing ENs, but also for the prediction of hazardous effects of new ENs that are being developed, thus enabling a sustainable growth of the nanotechnology-based industries.

Moreover, because the assessment of hazardous properties of ENs is a global concern, our NANOMMUNE consortium strives to harmonize toxicological testing and risk assessment efforts between Europe and the United States, through a balanced participation of investigators from EU member states (Sweden, Finland, Germany, United Kingdom), associated countries (Switzerland), and the United States. Reinforced international cooperation and sharing of data is of critical importance because a reliable basis for the assessment of safety of nanomaterial-based products and technologies requires the production and implementation of standardized test materials, toxicity assays, and risk assessment strategies.

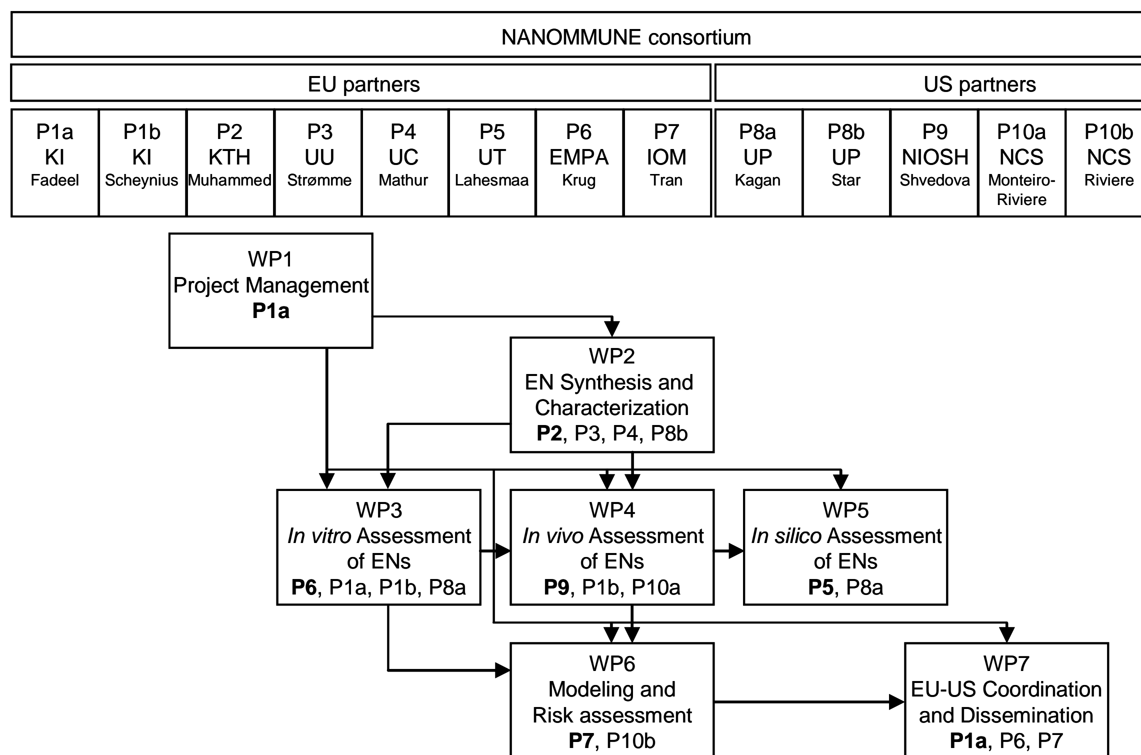
Impact, Quality Handbook

The multidisciplinary approach adopted in the NANOMMUNE consortium will contribute to the elucidation of the hazardous effects of ENs on the immune system, and will serve as a basis for reliable and sound assessment of the potential risks to human health posed by these new materials. NANOMMUNE will thus benefit a) *citizens*, because we address issues related to human health; b) *researchers*, because we will generate new knowledge in material production, and on mechanisms of interactions of nanomaterials with biological systems; and c) *industry* (including SME:s), through the incorporation of experimental protocols into a Quality Handbook (QHB), which can provide support to other interested parties.

Detailed standard operation procedures (SOPs) for the characterization methods (including sample preparation) have been established, and the protocols finally selected are included within the NANOMMUNE Quality Handbook (QHB). Taken together, our studies provide at the end a useful manual for other academic or industrial investigators and small companies who are interested in safe and standardized procedures for nanomaterial synthesis and handling.

The chapters of the Quality Handbook concern the workpackages WP02 (Material synthesis and characterisation), WP03 (in vitro Assessment), WP04 (in vivo Assessment) and WP05 (in silico Assessment, i.e. transcriptomics). Chapter 2 contains SOPs which are directly related to nanomaterials, their synthesis and characterisation, and therefore no special identification of “nano-specific” steps within the procedure have to be marked. However, for the other chapters this is needed [5]. We describe here a set of methods which may contain methodical steps which address specifically the nano-scale particulate matter or are sensitive to the physico-chemical properties of nanomaterials. Therefore, we labelled these specific steps with special symbols like ✋ (nano-sensitive step, interference of the materials with the analytical procedure likely) or ☝ (important step). Our aim is to deliver protocols which may be used for harmonisation between different European projects; these protocols can certainly be complemented step by step by future research

consortia. The history of publication in the field of Nanotoxicology teaches us that many of these publications contain false-positive or false-negative results based on methodological pitfalls and flaws. These have to be avoided in the future and with this Quality Handbook we want to take a step in this direction.



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2 Material Production and Characterisation

2.1 Material Production

2.1.1 SOP Nanommune 2.01_v3 - Synthesis of Cerium Oxide (CeO_2) Nanoparticles

2.1.1.1 Purpose

The purpose of this SOP is to synthesize Cerium Oxide (CeO_2) nanoparticles for various studies.

2.1.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptve procedure detailing the synthesis of synthesized Cerium Oxide (CeO_2) nanoparticles.

2.1.1.3 Principle

Cerium oxide (CeO_2) has shown that it is a potent antioxidant in cell culture models. Not only is cerium oxide used in biological applications, it also has and has shown to be a very good material in other applications such as Energy (1).

2.1.1.4 Reagents and Materials

- 500 mL flask
- 2X 250 buret
- 2 ring stand(s)
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- Other various glassware
- Analytical grade solution of Cerium (III) Nitrate, $Ce(NO_3)_3$ (Chempur 001175)
- Analytical grade Ammonium Hydroxide, NH_4OH.(VWR Lot# 09A300511)
- 12M Hydrochloric acid, HCl (Aldrich Lot#0001397813)
- Sodium Hydroxide, NaOH (Sigma Aldrich S5881-1kg)
- High purity water with a resistivity of 18MΩcm (Elga Purelab Option)
- Autoclave

2.1.1.5 *Safety procedures and precautions*

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.1.6 *Procedure* [(2)]

- Weigh specific amount of cerium (III) nitrate ($Ce(NO_3)_3$.6H2O), dissolve it in DI water and prepare 0.2 M $Ce(NO)_3$ solution using a volumetric flask.
- Mix specific amount of ammonia solution with DI water to prepare a 3 M NH_4OH solution.
- Add 25 ml 3 M NH_4OH solution into 50 ml 0.2 M $Ce(NO_3)_3$ solution with a vigorous stirring rate of 6500 rpm, a yellowish $Ce(OH)_3$ precipitate were formed immediately.
- Keep vigorous stirring the mixture for about 2 hours. Subsequently, the precipitate turns to purple, and finally become light yellow suspension.
- Centrifuge the suspension to separate the precipitate, and wash the precipitate with DI water and ethanol for three times respectively, then dry at 120°C overnight.
- Calcination was performed on the dried precursor in a box furnace at 200°C under air, and a pale yellow powder of CeO_2 was thus obtained.
- The particles are allowed to cool. Afterwards, the particles are transferred to the autoclave with 35mL of 2M NaOH.
- The final product was placed into a beaker and titrated with 12 M HCl until the pH was 7.
- The particles were collected by use of filtration.
- After filtration, the particles were allowed to dry in the vacuum oven (Vacucell).

2.1.1.7 *References*

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2.1.2 SOP Nanommune 2.2_v2 - Synthesis of Dextran coated Magnetite (Fe_3O_4) Nanoparticles

2.1.2.1 Purpose

To synthesize dextran coated magnetite (Fe_3O_4) nanoparticles for various studies.

2.1.2.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of synthesize dextran coated magnetite (Fe_3O_4) nanoparticles.

2.1.2.3 Principle

Surface coated magnetic iron oxide nanoparticles are of interest. Therefore; dextran-magnetite complex is a biocompatible magnetic fluid. This particular complex was developed as a new medical agent, which is applicable to magnetic resonance imaging and hypothermia.

2.1.2.4 Reagents and Materials

- 500 mL 3 neck bottle flask
- Glycerol bath
- Nitrogen gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- Other various glassware
- Analytical grade solution of $FeCl_2$ (Sigma Aldrich 877002-149 250g)
- Analytical grade $FeCl_3$ (Sigma Aldrich 44944-250g)
- 12M Hydrochloric acid, HCl (Aldrich Lot#0001397813)
- Sodium Hydroxide, NaOH (Sigma Aldrich S5881-1kg)
- High purity water with a resistivity of 18MΩcm (Elga Purelab Option)
- Dextran 70,000da (Sigma PC 52809166 31390-25g)
- Dextran 6,000da (Sigma PC 32409217 31388-25g)
- Dextran 40,000da (Sigma PC 22909060 31389-25g)

2.1.2.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous

- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.2.6 Procedure KTH-Dextran (MW= 6,000, 40,000 and 70,000) Coated Fe_3O_4-Labmade

- A stock solution of iron (III) and iron (II) in chloride media was prepared by dissolving the respective iron hydrated precursors Fe^{+3}(1M)/Fe^{+2} (0.5M) with a deoxygenated 0.1M HCl aqueous solution.
- This solution was heated by purging the nitrogen gas continuously.
- Once the reaction temperature reached to 70°C, 0.7 M NH_4OH was added to the deoxygenated solution under constant stirring rate at 250 rpm.
- NaOH was added to the flask when the T= 70°C.
- The stirring was kept for about 45 minutes and the particles were decantated by magnetic settling.
- The obtained particles were washed with deoxygenated water for three times.
- A stable ferrofluid was prepared by dispersing the particles in a 0.01M TMAOH aqueous
- After the third wash, 45ml of magnetite nanoparticles was placed in a beaker with 10.88g of Dextran.
- Place on the Multi-Wrist Shaker for 24 hours at 6rpm.
- The final product was placed into Spectra Pro MWCO 25,000 bag (MW= 6,000) and MWCO bag 100,000 (Mw = 40,000 and 70,000) for dialysis for 3 days, while changing the water every 3 hours.
- After dialysis has been completed, place product in container for storage.

2.1.2.7 Another method for KTH-Dextran-SPION-Labmade

- The obtained particles were washed with deoxygenated water for three times.
- A stable ferrofluid was prepared by dispersing the particles in a 0.01M TMAOH aqueous solution.
- After the third wash, dissolve exact amount of dextran (MW=40,000 6,000 70,000) in deionized water.
- According to the literatures the highest Mw of dextran will produce smaller hydrodynamic size.
- After the formation of Fe_3O_4 the dextran was added to the mixture and stirred for 30min. at T=70°C.

- Washed three consecutive times and collected the particles.

2.1.2.8 References

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2.1.3 SOP Nanommune 2.03_v2 - Synthesis of Magnetite (Fe_3O_4) Nanoparticles

2.1.3.1 Purpose

The purpose of this SOP is to synthesize magnetite (Fe_3O_4) nanoparticles for various studies.

2.1.3.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of synthesize magnetite (Fe_3O_4) nanoparticles .

2.1.3.3 Principle

Magnetic iron oxide nanoparticles; especially those particles that are surface functionalized are a novel functional material which has been widely used in various different venues such as: biotechnology, data storage, catalysis, and magnetic fluids.

2.1.3.4 Reagents and Materials

- 500 mL 3 neck bottle flask
- Glycerol bath
- Nitrogen gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- Other various glassware
- Analytical grade solution of FeCl2 (Sigma Aldrich 877002-149 250g)
- Analytical grade $FeCl_3$ (Sigma Aldrich 44944-250g)
- 12M Hydrochloric acid, HCl (Aldrich Lot#0001397813)
- Sodium Hydroxide, NaOH (Sigma Aldrich S5881-1kg)
- High purity water with a resistivity of 18MΩcm (Elga Purelab Option)

2.1.3.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.3.6 *Procedure*[1]

- A stock solution of iron (III) and iron (II) in chloride media was prepared by dissolving the respective iron hydrated precursors Fe^{+3} (1M)/Fe^{+2} (0.5M) with a deoxygenated 0.1 M HCl aqueous solution.
- This solution was heated by purging the nitrogen gas continuously.
- Once the reaction temperature reached to 70°C, 0.7 M NH_4OH was added to the deoxygenated solution under constant stirring rate at 250 rpm.
- NaOH was added to the flask when the T= 70°C.
- The stirring was kept for about 45 minutes and the particles were decantated by magnetic settling.
- The obtained particles were washed with deoxygenated water for three times.
- A stable ferrofluid was prepared by dispersing the particles in a 0.01M TMAOH aqueous solution.

2.1.3.7 *References*

1. C.C. HUA, S. ZAKARIA, R. FARAHIYAN, L. T. KHONG, K. L. NGUYEN, M. ABDULLAH and S. AHMAD, Sains Malaysiana. 37 (2008), 389-394.

2.1.4 SOP Nanommune 2.10_v1 - Synthesis of Mesoporous material UU-AMS-6 as synthesized

2.1.4.1 Purpose

To synthesize mesoporous particles of cubic porous structure, with space group Ia3d, known as AMS-6_as-synthesised. This material is non-porous as it contains the surfactant still within its pores.

2.1.4.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.4.3 Principle

The preparation of mesoporous material AMS-6 relies in the cooperative self assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a "binding" agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves three stages; (i) assembly, (ii) particle growth.

2.1.4.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Alanine (C_{12}AlaA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm
- Filter papers, and filtering equipment (Sigma-Aldrich)
- A calcination oven

2.1.4.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.4.6 Procedure

2.1.4.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Alanine (C_{12}AlaA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}AlaA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.4.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C_{12}Ala: APES: TEOS: H2O 1: 1.25: 6.7: 309.1.
- The resultant material is known as AMS-6_as synthesized.

2.1.4.7 References

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813

[2] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.

[3] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

2.1.5 SOP Nanommune 2.11_v1 - Synthesis of Mesoporous material UU-AMS-6 calcined

2.1.5.1 Purpose

To synthesize mesoporous particles of cubic porous structure, with space group Ia3d, known as AMS-6_calcined.

2.1.5.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.5.3 Principle

The preparation of mesoporous material AMS-6 relies in the cooperative self-assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a "binding" agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves three stages; (i) assembly, (ii) particle growth and (iii) calcination of the surfactant to form the porous material.

2.1.5.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Alanine (C_{12}AlaA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm
- Filter papers, and filtering equipment (Sigma-Aldrich)
- A calcination oven

2.1.5.5 *Safety procedures and precautions*

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.5.6 *Procedure*

2.1.5.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Alanine (C_{12}AlaA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}AlaA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.5.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C_{12}Ala: APES: TEOS: H2O 1: 1.25: 6.7: 309.1.
- The resultant material is known as AMS-6_as synthesized.

2.1.5.6.3 Calcination

- The surfactant is removed by thermal treatment in an oven at 550 ^{o}C in a stream of nitrogen followed by oxygen in order to remove the organic surfactant.
- The resultant material is known as AMS-6_calcined and possesses no organic functional groups.

2.1.5.7 *References*

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813

[2] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.

[3] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

2.1.6 SOP Nanommune 2.12_v1 - Synthesis of Mesoporous material UU-AMS-6 extracted

2.1.6.1 Purpose

To synthesize mesoporous particles of cubic porous structure, with space group Ia3d, known as AMS-6_extracted. This nanomaterial possess a surface coverage of propyl amine groups within the internal pore space, which are covalently bound to the silica wall.

2.1.6.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.6.3 Principle

The preparation of mesoporous material AMS-6 relies in the cooperative self assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a “binding” agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves three stages; (i) assembly, (ii) particle growth and (iii) extraction of the surfactant to form the propyl-amine functionalized porous material.

2.1.6.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Alanine (C_{12}AlaA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm
- Filter papers, and filtering equipment (Sigma-Aldrich)

- Ethanol.

2.1.6.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.6.6 Procedure

2.1.6.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Alanine (C_{12}AlaA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}AlaA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.6.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C_{12}Ala: APES: TEOS: H2O 1: 1.25: 6.7: 309.1.
- The resultant material is known as AMS-6_as synthesized.

2.1.6.6.3 Calcination

- The surfactant is removed by solvent extraction by refluxing the as-synthesized product in an ethanol solution for a period of 12 hours.
- The resultant material is filtered and dried at ambient temperatures, and is known as AMS-6_extracted and possesses.

2.1.6.7 References

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813

[2] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.
[3] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

2.1.7 SOP_2.15 - Synthesis of Oleate Capped Titanium Oxide (TiO_2) Nanorods _v3

2.1.7.1 Purpose

To synthesize oleate capped Titanium Oxide (TiO_2) nanorods soluble in organic solvents for further surface modification. Nanorods are 3 nm in diameter and 40 nm in length.

2.1.7.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of synthesized Titanium Oxide (TiO_2) nanorods.

2.1.7.3 Principle

Anatase Titanium Oxide nanorods are synthesized by a heating up method using a molecular precursor(1).

2.1.7.4 Reagents and Materials

- Oleic acid
- 1-Octadecene
- $Ti(OiPr)_4$
- Oleylamine
- Acetone
- Hexane
- 50 ml three necked flask and various classware
- Schlenk line and inert gas supply
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge

2.1.7.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood
- Lab coats, gloves and glasses must be worn at all times

2.1.7.6 Procedure

- Flask must be dry and inert.
- Add oleic acid (1.6 ml, 5 eq) and 1-octadecene (6ml) under nitrogen and degas for 30 minutes under high vacuum.
- Add reflux condenser and heat up to 80°C under gentle nitrogen gas flow.
- Add Ti(OiPr)4 and hold temperature for 20 minutes while the mixture is stirring.
- Heat up to 260°C and hold temperature for 10 minutes.
- Add oleylamine (0.32 ml, 1 eq) quickly with a syringe while heavily stirring and hold temperature for 1 hour. After that let the mixture cool down to room temperature.
- Add 20 ml of hexane to the reaction mixture at room temperature.
- Add 40 ml of acetone to precipitate particles and centrifuge until the centrifuge effluent becomes totally clear.
- Resolve particles in 10 ml of hexane and precipitate with 80 ml of Acetone with subsequent centrifugation. Repeat this procedure for at least three times.
- Store derived nanorods resolved in Hexane at -15°C.

2.1.7.7 References

(1.) ZHANG et al, Angew. Chem.117 (2005), 3532 – 3536.

2.1.8 SOP 2.16 - Synthesis of Mesoporous material UU-AMS-8 as synthesized_v1

2.1.8.1 Purpose

To synthesize mesoporous particles of cubic porous structure with mesocaged porosity, with space group Fd3m, known as AMS-8_as-synthesised. This material is non-porous as it contains the surfactant still within its pores.

2.1.8.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.8.3 Principle

The preparation of mesoporous material AMS-8 relies in the cooperative self assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a “binding” agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves three stages; (i) assembly, (ii) particle growth.

2.1.8.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Glutamic (C_{12}GlutA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm
- Filter papers, and filtering equipment (Sigma-Aldrich)
- A calcination oven.

2.1.8.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.8.6 Procedure

2.1.8.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Glutamic acid (C_{12}GlutA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}AGlutA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.8.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C_{12}GlutA: APES: TEOS: H_2O 0.1: 0.1: 1: 155.
- The resultant material is known as AMS-8_as synthesized.

2.1.8.7 References

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813.

[2] A. E. Garcia-Bennett, K. Miyasaka, O. Terasaki, Chem. Mater., 16 (2004) 3597.

[3] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.

[4] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

2.1.9 SOP_2.17 - Synthesis of Mesoporous material UU-AMS-8 calcined_v1

2.1.9.1 Purpose

To synthesize mesoporous particles of cubic porous structure with mesocaged porosity, with space group Fd3m, known as AMS-8_calcined. This material is mesoporous as it contains the surfactant still within its pores.

2.1.9.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.9.3 Principle

The preparation of mesoporous material AMS-8 relies in the cooperative self assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a "binding" agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves three stages; (i) assembly, (ii) particle growth, (iii) Calcination.

2.1.9.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Glutamic (C_{12}GlutA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm
- Filter papers, and filtering equipment (Sigma-Aldrich)
- A calcination oven.

2.1.9.5 *Safety procedures and precautions*

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.9.6 *Procedure*

2.1.9.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Glutamic acid (C_{12}GlutA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}AGlutA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.9.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C_{12}GlutA: APES: TEOS: H2O 0.1: 0.1: 1: 155.
- The resultant material is known as AMS-8_as synthesized.

2.1.9.6.3 Calcination

- The surfactant is removed by thermal treatment in an oven at 550 ^{o}C in a stream of nitrogen followed by oxygen in order to removed the organic surfactant.
- The resultant material is known as AMS-8_calcined and possesses no organic functional groups.

2.1.9.7 *References*

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813.

[2] A. E. Garcia-Bennett, K. Miyasaka, O. Terasaki, Chem. Mater., 16 (2004) 3597.

[3] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.

[4] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

2.1.10 SOP_2.18 - Synthesis of Mesoporous material UU-AMS-8 as extracted_v1

2.1.10.1 Purpose

To synthesize mesoporous particles of cubic porous structure with mesocaged porosity, with space group Fd3m, known as AMS-8_extracted. This material contains mesocage and a layer of functionalized amine groups resulting from the extraction of the surfactant.

2.1.10.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.10.3 Principle

The preparation of mesoporous material AMS-8 relies in the cooperative self assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a "binding" agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves three stages; (i) assembly, (ii) particle growth, (iii) Extraction/Calcination.

2.1.10.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Glutamic (C_{12}GlutA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm
- Filter papers, and filtering equipment (Sigma-Aldrich)
- A calcination oven.

2.1.10.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.10.6 Procedure

2.1.10.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Glutamic acid (C_{12}GlutA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}AGlutA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.10.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C_{12}GlutA: APES: TEOS: H2O 0.1: 0.1: 1: 155.
- The resultant material is known as AMS-8_as synthesized.

2.1.10.6.3 Extraction/Calcination

- The surfactant is removed by solvent extraction by refluxing the as-synthesized product in an ethanol solution for a period of 12 hours.
- The resultant material is filtered and dried at ambient temperatures, and is known as AMS-8_extracted and possesses.

2.1.10.7 References

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813.

[2] A. E. Garcia-Bennett, K. Miyasaka, O. Terasaki, Chem. Mater., 16 (2004) 3597.

[3] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.

[4] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

2.1.11 SOP_2.19 - Synthesis of Mesoporous material UU-AMS-8FITC_v1

2.1.11.1 Purpose

To synthesize functionalized mesoporous particles of cubic porous structure, with space group Fd3m, known as AMS-8_extracted and to use the amine moieties of the functional groups in order to form imminothioester bonds to fluorescein isothiocyanate (here called FITC). The isothiocyanate group of the fluorophore is responsible for the covalent binding; hence it doesn't affect the fluorescent properties of the bonded molecule.

2.1.11.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis and hydrothermal treatments in order to prepare batches of up to 50 grams of material.

2.1.11.3 Principle

The preparation of mesoporous material AMS-8 relies in the cooperative self assembly of surfactant micelles, specifically anionic surfactant micelles, together with two silica sources, namely; APES (3-aminopropyl triethoxy silane), and TEOS (Tetraethyl orthosilicate). The latter is used as a "binding" agent or co-structure directing agent between the organic surfactant and the inorganic wall, and the former as the silica source for the inorganic silica wall. The procedure involves four stages; (i) assembly, (ii) particle growth, (iii) extraction of the surfactant to form the propyl-amine functionalized porous material, and (iv) reaction with FITC.

2.1.11.4 Reagents and Materials

- 500 mL 3 neck PPT bottle flask
- Glycerol bath or heating bath
- Air gas
- Disposable plastic pipettes
- Magnetic and Teflon Stirrers
- Mass balance
- Thermometer
- 3-Aminopropyl triethoxysilane (Sigma-Aldrich)
- Tetraethyl orthosilicate (Sigma-Aldrich)
- N-Lauroyl-L-Glutamic (C_{12}GlutA) surfactant (Nanologica AB, Sweden)
- High purity water with a resistivity of 18MΩcm

- Filter papers, and filtering equipment (Sigma-Aldrich)
- Ethanol.
- Methanol.
- Sodium hydroxide pellets (Sigma-Aldrich)
- Fluorescein isothiocyanate (Sigma-Aldrich)

2.1.11.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times

2.1.11.6 Procedure

2.1.11.6.1 Self-Assembly

- The anionic surfactant used is N-Lauroyl-L-Glutamic acid (C_{12}GlutA) (Nanologica AB, Sweden).
- The co-structure directing agent 3-aminopropyl triethoxysilane (APES, Sigma-Aldrich) and tetraethyl orthosilicate (TEOS, Sigma-Aldrich) are used as silica sources.
- All chemicals can be used as received.
- A homogenous solution of surfactant C_{12}GlutA was obtained in distilled water at 80ºC for 24h under static conditions.
- The surfactant solution was stirred for 10min before addition of APES; TEOS.

2.1.11.6.2 Particle Growth

- The synthesis gel was subsequently stored at room temperature under stirring conditions for 24h.
- The solid product was filtered and dried at RT and under atmospheric pressure conditions.
- The molar composition of the reaction mixtures was C12GlutA: APES: TEOS: H2O 0.1: 0.1: 1: 155.
- The resultant material is known as AMS-8_as synthesized.

2.1.11.6.3 Solvent Extraction

- The surfactant is removed by solvent extraction by refluxing the as-synthesized product in an ethanol solution for a period of 12 hours.

- The resultant material is filtered and dried at ambient temperatures, and is known as AMS-8_extracted and possesses.

2.1.11.6.4 (iv) Reaction with FITC

- Extracted (amine functionalized) AMS-8 particles (1 gram) are refluxed for 24h in methanol containing the desired amount of fluorochrome (typically in excess) at pH 11 (obtained by addition of NaOH).
- The remaining orange solid is then filtered, washed with distilled (250 ml) water, and ethanol (100ml) and left to dry at 60°C under ambient conditions. The resulting material is known as AMS-8_FITC.
- AMS-8_FITC must be stored in a dark container to prevent bleaching of the flurochrome with light.

2.1.11.7 References

[1] A. E. Garcia-Bennett, S. Che, T. Tatsumi, O. Terasaki, Chem. Mater., 16 (2004) 813

[2] S. Che, A. E. Garcia-Bennett, X. Liu, R. P. Hodgkins, P. A. Wright, D. Zhao, O.Terasaki, T. Tatsumi Angewandte Chemie Int. Ed., 2003, 42, (33), 3930.

[3] S. Che, A. E. Garcia-Bennett, T. Yokoi, K. Sakamoto, H. Kunieda, O. Terasaki, T. Tatsumi, Nature Mater., 2003, 2, 801.

[4] E. Witasp, N. Kupferschmidt, L. Bengtsson, K. Hulternby, C. Smedman, S. Paulie, A. E. Garcia-Bennett and B. Fadeel, Toxicology and Applied Pharmacology, 2009, 239 (3) 306.

[5] A. E. Garcia-Bennett, K. Miyasaka, O. Terasaki, Chem. Mater., 16 (2004) 3597.

[6] H. Vallhov, S. Gabrielsson, M. Strømme, A. Schenynus, A. E. Garcia-Bennett Nanoletters, 2007, 7 (12), 3576 -3582.

2.1.12 SOP_2.20 - Synthesis of Bare Zinc Oxide (ZnO) Nanoparticles_v2

2.1.12.1 Purpose

To synthesize Zinc Oxide (ZnO) nanoparticles . Nanoparticles are ca. 15 nm in diameter.

2.1.12.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of synthesized Titanium Oxide (TiO_2) nanorods.

2.1.12.3 Principle

Zink Oxide nanoparticles are synthesized by a solvothermal method using a molecular precursor(1).

2.1.12.4 Reagents and Materials

- $Zn(acetate)_2 \cdot 2H_2O$
- Methanol
- KOH
- 250 ml flask and various class ware
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Ultrasonic bath

2.1.12.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times

2.1.12.6 Procedure

- Resolve 3.2 g of $Zn(acetate)_2 \cdot 2H_2O$ (0.015 M) in 187.5 ml of Methanol in a 250 ml flask and heat to 60°C. Reaction mixture is stirred for 10 minutes at this temperature.
- Resolve 2.52 g of KOH (0.045 M) in 42.5 ml of Methanol (35% less solvent then described in reference (1)) in another 100 ml flask and stir at 60°C for 10 minutes.

- Add the second solution to the first one during 30 sec. The solution is getting turbid until it is getting clear again after 5 min.
- Add reflux condenser and reflux the reaction mixture at 60°C for three hours.
- Use the rotary evaporator to remove most of the Methanol.
- Cleaning: Add 80 ml of a 1:1 mixture of Methanol and water. Sonicate for 10 minutes and centrifuge for 15 min. Afterwards wash the particles three times with 80 ml of water.
- Freeze-thaw dry particles.

2.1.12.7 References

1. WELLER et al., Angew. Chem. Int. Ed. 41 (2002), 1189 – 1191

2.1.13 SOP_2.21 - Synthesis of iron oxide core – silica shell nanoparticles_v1

2.1.13.1 Purpose

To prepare iron oxide core – silica shell nanoparticles with various overall sizes.

2.1.13.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of iron oxide core – silica shell nanoparticles with three overall sizes 30 nm, 50 nm and 120 nm utilising two methods (micromulsion and Stöber).

2.1.13.3 Principle

The iron oxide nanoparticles, produced by thermal decomposition of iron oxide hydroxide (FeO(OH)) are undergoing a phase transfer process by ligand exchange mechanism in the microemulsion system. This will result in the transfer of the hydrophobic iron oxide to the water phase (water droplets in suspension in the dominant oil phase) and successive coating with a silica layer. The thickness of the silica layer is time dependent, the quantity of the precursor being maintained constant. To ensure the formation of single core core – shell nanoparticles an optimisation of the ratios between the reactants and the concentration of the iron oxide core has been performed. In addition, in order to maintain the monodispersity of the obtained particles, a multistep washing post synthesis process was carried out.

2.1.13.4 Reagents and Materials

- 250 ml 3 neck round bottom flask, 25 ml round bottom flask
- centrifugal stirrer of 40 mm diameter of the paddles
- mechanical stirrer
- cyclohexane (99.5%)
- hexanol (98%)
- Triton X100 (analytical grade)
- Deionised water (DI water)
- NH_4OH (28%)
- Iron oxide suspension in cyclohexane
- TEOS (99.5%)
- ethanol (99.9%)
- 5% nitric acid
- 50 ml plastic tubes

- 15 ml plastic tubes

2.1.13.5 *Safety procedures and precautions*

- Standard safety operating procedures and rules of working in a chemistry lab are to be followed at all times
- Treat all chemicals as dangerous, read the safety instructions in their data sheets prior starting working with them
- All procedures (unless otherwise stated) are to be performed in the fumehood
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

2.1.13.6 *Procedure for preparation of core – shell nanoparticles with the overall sizes of 30 nm and 50 nm*

2.1.13.6.1 Preparation of iron oxide suspension

- Disperse the right volume of iron oxide suspension of stock solution in cyclohexane to get the final concentration of iron oxide 0.19 mg/ml
- For better dispersion of the particles let the suspension to sonicate for 5 minutes

2.1.13.6.2 Microemulsion preparation

- In a 250 ml 3 necks round bottom flask add the following chemicals according to the table below:

Chemicals Used	Quantity (mols)
H_2O	0.14
Hexanol	0.05
NH_4OH (23%)	0.0065
Triton X100	0.025

- Insert the stirrer inside and connect it to the mechanical stirrer.
- Start the mixing of the water phase components with the oil phase (containing the iron oxide nanoparticles) under stirring.
- The reaction is conducted at the room temperature under continuous stirring at the stirring rate of 1000 rpm

2.1.13.6.3 Silica layer formation

- After the formation of the microemulsion, 0.7 mmols of silica precursor (TEOS) is added and the stirring is continued for the desired time according with the thickness of the silica layer to be achieved.

2.1.13.6.4 The separation of the silica coated iron oxide from the microemulsion

- Change the pH of the microemulsion to 1-2 by adding drop by drop a solution of 5% nitric acid

- Stop the mechanical stirring
- Transfer the microemulsion to 50 ml plastic tubes
- Cool rapidly the suspension by dipping the tubes in liquid N_2 for 2-3 min (A)
- Centrifuge the suspension in cycles of 6-7 min at 5000 rpm for small particles (27 nm) and at 3500 rpm for medium size particles (50 nm).(B)
- Transfer the upper part of the suspensions (in which the particles are concentrated) in a separated tube (C)
- Repeat the steps from (A) to (C) till the down part of the microemulsion is reasonably depleted of particles.

2.1.13.6.5 The particles washing

- Transfer the collected particles in 15 ml tubes
- Fill in approximately half of the tubes
- Add 99.9% ethanol till 15 ml
- Cool rapidly the suspension by dipping the tubes in liquid N_2 for 2-3 min(A)
- Centrifuge the suspension in cycles of 10 min at 8000 - 9000 rpm (B)
- Transfer the upper part of the suspension in new 15 ml tubes (C)
- The collected particles at the bottom of the tubes are resuspended in 99.9% ethanol and dispersed by sonication for 1 min.
- Repeat the steps from (A) to (C). until the ethanol suspension is reasonably depleted of particles.
- Repeat the steps 1 to 8 three times.

2.1.13.6.6 The transfer of the particles to the water phase

- 6.6.1. After the particles are washed three times with 99.9% ethanol the final collected particles are transferred in 15 ml tubes and a solution of 15% water in ethanol (v/v) is added till 15 ml.
- 6.6.2. Cool rapidly the suspension by dipping the tubes in liquid N2 for 2-3 min
- 6.6.3. Centrifuge the suspension in cycles of 10 min at 8000 - 9000 rpm
- 6.6.4. Transfer the upper part of the suspension in new 15 ml tubes
- 6.6.5. The collected particles at the bottom of the tubes are resuspended in a solution of 25% water in ethanol (v/v) and dispersed by sonication for 1 min
- 6.6.6. Repeat the steps from 6.6.2. to 6.6.5. until the suspension is reasonably depleted of particles.

- 6.6.7. The collected particles from the above washing steps are transferred in 15 ml tubes and a solution of 25% water in ethanol (v/v) is added till 15 ml.
- 6.6.8. Repeat the steps from 6.6.2. to 6.6.4.
- 6.6.9. The collected particles at the bottom of the tubes are resuspended in a solution of 50% water in ethanol (v/v) and dispersed by sonication for 1 min.
- 6.6.10. Repeat the steps from 6.6.2. to 6.6.5. until the suspension is reasonably depleted of particles.
- 6.6.11. The collected particles from the above washing steps are transferred in 15 ml tubes and a solution of 50% water in ethanol (v/v) is added till 15 ml.
- 6.6.12. Repeat the steps from 6.6.2. to 6.6.4.
- 6.6.13. The collected particles at the bottom of the tubes are resuspended in a solution of 75% water in ethanol (v/v) and dispersed by sonication for 1 min.
- 6.6.14. Repeat the steps from 6.6.2. to 6.6.5. until the suspension is reasonably depleted of particles.
- 6.6.15. The collected particles from the above washing steps are transferred in 15 ml tubes and a solution of 75% water in ethanol (v/v) is added till 15 ml.
- 6.6.16. Repeat the steps from 6.6.2. to 6.6.4.
- 6.6.17. The collected particles at the bottom of the tubes are resuspended in DI water and dispersed by sonication for 1 min.
- 6.6.18. Repeat the steps from 6.6.2. to 6.6.5. until the suspension is reasonably depleted of particles.
- 6.6.19. The collected particles from the above washing steps are transferred in a 15 ml tubes and the desired volume of DI water is added.

2.1.13.7 Procedure for preparation of core – shell nanoparticles with the overall sizes of 120 nm

2.1.13.7.1 Silica layer growing

- In a 25 ml round bottom flask add the following chemicals according to the table below:

Chemicals Used	*Volume (ml)*
H_2O	6
Ethanol	9
NH_4OH (28%)	0.3
TEOS	$175x10^{-3}$
30 nm core – shell nanoparticles in ethanol	0.35

- Insert the stirrer inside and connect it to the mechanical stirrer.
- Start the mixing of the components by stirring.

- The reaction is conducted at the room temperature under continuous stirring at the stirring rate of 1000 rpm for 3 h at room temperature to obtain a silica shell thickness of 45 nm.

2.1.13.7.2 The particles washing

- 7.2.1 After the silica condensation process is completed for the desired thickness of silica shell the stirring is stopped and the particles are transferred in 2x15 ml tubes
- 7.2.2. Add 99.9% ethanol till 15 ml
- 7.2.3. Cool rapidly the suspension by dipping the tubes in liquid N_2 for 2-3 min
- 7.2.4. Centrifuge the suspension in cycles of 10 min at 8000 - 9000 rpm
- 7.2.5. Transfer the upper part of the suspension in new 15 ml tubes
- 7.2.6. The collected particles at the bottom of the tubes are resuspended in DI water and dispersed by sonication for 1 min.
- 7.2.7. Repeat the steps from 7.2.3. to 7.2.6. until the ethanol suspension is reasonably depleted of particles.
- 7.2.8. The collected particles from the above washing steps are transferred in 15 ml tubes and DI water is added till 15 ml.
- 7.2.9. Repeat the steps from 7.2.2. to 7.2.8. three times (the ethanol 99.9% is replaced by DI water).
- 7.2.10. The collected particles from the above washing steps are transferred in a 15 ml tubes and the desired volume of DI water is added.

2.1.13.7.3 Preservation conditions and handling precautions

- The particles synthesised as described above should be kept in the fridge (4°C) until they are used.
- Precautions should be taken to avoid the evaporation of the solvent that would result in changes in the concentration of the particles in suspension. For this purpose it is recommended a tight sealing of the cap of the tubes and the application of parafilm around the caps.
- Just prior using the particles they should be put in an ultrasonic bath at 4°C (with ice cubes) for 1 minute for a homogeneous redispersion of the core – shell particles in the solvent.
- In case the transportation of the particles suspensions is needed care should be taken that the temperature of the samples is maintained low (< 40°C).

2.1.13.8 Reference

(1) Carmen Vogt, Muhammet S. Toprak, Mamoun Muhammed, Sophie Laurent, Jean-Luc Bridot, Robert N. Müller, High quality and tuneable silica shell-magnetic core nanoparticles, Journal of Nanoparticles Research, DOI:10.1007/s11051-009-9661-7, http://www.springerlink.com/content/gp7237771pv6505w

2.1.14 SOP_2.28 - Synthesis of Graphene Oxide_v1

2.1.14.1 Purpose

To synthesize graphene oxide for various studies, where this nanomaterial can be applied in aqueous environments.

2.1.14.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of graphene oxide.

2.1.14.3 Principle

Graphene oxide is an oxidized precursor of chemically converted graphene, a scalable alternative for graphene. Graphene oxide nanoparticles have been suggested for cellular imaging and drug delivery vehicles for biological applications.

2.1.14.4 Reagents and Materials

- 50 mL round bottom flask
- 1 L round bottom flask
- 1 medium sized magnetic stir bar
- 2, 24/40 rubber septum
- 2, 18G needle
- 150 mL, coarse fritted-glass filter
- Disposable Pasteur pipettes
- Branson Bath Ultrasonicator Model 5510 (Frequency 40 kHz)
- Magnetic stirrer/hot plate
- 2, 250 mL polypropylene Oak Ridge centrifuge tubes
- Sorvall RC-5C Plus Centrifuge with Sorvall SLA-1500 rotor
- Vacuum oven
- Oil bath
- Ice bath
- Mass balance
- Thermometer
- Sample vials (20 mL)
- Graduated cylinder
- Analytical grade sulfuric acid (H_2SO_4)

- Analytical grade hydrochloric acid (HCl)
- 30% hydrogen peroxide (H_2O_2)
- Sodium bicarbonate ($NaHCO_3$)
- Graphite flakes (332461, from Sigma Aldrich)
- Potassium persulfate (K2S2O8, 216224, from Sigma Aldrich)
- Phosphorus pentoxide (P_2O_5, P0679, from Sigma Aldrich)
- Potassium permanganate ($KMnO_4$, P0679, from Sigma Aldrich)
- High purity water
- Spectra/Por dialysis membrane (7, MWCO 1000)
- Universal dialysis tubing closures

2.1.14.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- $K_2S_2O_8$/P_2O_5/$KMnO_4$/H_2SO_4/HCl/H_2O_2 (30%) are highly oxidizing. Caution must be taken when handling these materials.
- All procedures (unless otherwise stated) are to be performed in a fume hood.
- Lab coats, latex gloves, and goggles must be worn at all times.

2.1.14.6 Procedure

2.1.14.6.1 Pre-oxidation of graphite flakes

- Measure 6.0 mL of H_2SO_4 and pour into 50 mL flask.
- Weigh out 2.0 g of $K_2S_2O_8$ and place in 50 mL round bottom flask.
- Weigh out 2.0 g of P_2O_5 and place in 50 mL round bottom flask.
- Cap round bottom flask with a 24/40 septum and immediately puncture the septum with an 18-gauge needle to release pressure.
- Heat content of 50 mL round bottom flask to 80°C using hot plate/oil bath with stirring. Verify temperature of bath with thermometer.
- Once $K_2S_2O_8$ and P_2O_5 dissolve, weigh out 2.0 g of graphite flakes and place in 50 mL round bottom flask
- Recap round bottom flask with a 24/40 septum and immediately puncture the septum with an 18-gauge needle to release pressure.
- Allow cooling to room temperature over a period of 6 hours.

- Dilute the mixture with high purity water, filter using a 150 mL, coarse fritted-glass filter, and wash on the filter until the rinse water pH became neutral. The filtrate should be neutralized with $NaHCO_3$.
- Air-dry the pre-oxidized graphite flakes at ambient temperature.

2.1.14.6.2 Oxidation employing modified Hummers' method

- Add 50 mL of H_2SO_4 to the 1 L flask.
- Cool the H_2SO4 to 0°C using an ice bath.
- Add 2.0 g of pre-oxidized graphite powder.
- Gradually add 6.0 g of $KMnO_4$ with stirring on the ice bath while ensuring that the temperature of the mixture does not reach 20°C.
- Cap round bottom flask with a 24/40 septum and immediately puncture the septum with an 18-gauge needle to release pressure.
- Stirred at 35°C for 2 hours using hot plate and oil bath.
- Add 92.0 mL of high purity water
- Cap round bottom flask with a 24/40 septum and immediately puncture the septum with an 18-gauge needle to release pressure.
- Stir for 15 minutes
- Add 280 mL of high purity water
- Add 5.0 mL of 30% H_2O_2 solution (the color of the mixture should change to bright yellow)
- Pour content into 2, 250 mL polypropylene Oak Ridge centrifuge tubes ensuring that the weight is even.
- Centrifuge solution at 8,000 r.p.m for 10 min.
- Decant the supernatant. The supernatant was neutralized with $NaHCO_3$.
- Thoroughly wash the precipitate in each Oak Ridge tube with 250 mL of a mixture of 1:10 $HCl:H_2O$. Ensure that the weight in each Oak Ridge tube is even.
- Centrifuge solution at 3,400 r.p.m. for 10 min.
- Decant the supernatant. The supernatant was neutralized with $NaHCO_3$.
- Repeat steps 6.25-6.27 (Numbers do not exist in original version) two additional times (1.5 L total 1:10 $HCl:H_2O$ mixture volume).
- Thoroughly wash the precipitate in each Oak Ridge tube with 100 mL of high purity H_2O. Ensure that the weight in each Oak Ridge tube is even.
- Centrifuge solution at 3,400 r.p.m. for 10 min.

- Discard the supernatant.
- Repeat steps 6.29-6.30 three additional times collecting the supernatant (i.e. graphite oxide).
- Dialyze graphite oxide against high purity water for 7 days changing the water on a daily basis.

2.1.14.6.3 Graphene oxide exfoliation

- Create a mixture of 1:3 dialyzed graphite oxide to high purity water.
- Sonicate mixture for 30 minutes employing the ultrasonicator bath.
- Centrifuge for 30 minutes at 3,400 r.p.m. to remove any unexfoliated graphite oxide.
- Transfer supernatant to vials.
- Dry water under vacuum conditions.
- Scrape product off with a spatula into a sample vial.

2.1.15 SOP 2.30 Synthesis of UC-ZnO_15-1_v1 - Synthesis of Bare Zinc Oxide (ZnO) Nanoparticles

2.1.15.1 Purpose

To synthesize bare zinc oxide (ZnO) nanoparticles. Nanoparticles are ca. 8nm diameter.

2.1.15.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of ZnO ENs.

2.1.15.3 Principle

Zink oxide nanoparticles were synthesized by a solvothermal method using zinc acetate as precursor (1).

2.1.15.4 Reagents and Materials

- $Zn(CH_3COO)_2 \cdot 2H_2O$
- CH_3OH
- KOH
- 250 ml flask and various glass ware
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Ultrasonic bath

2.1.15.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.1.15.6 Procedure

- Dissolve 3.2 g (15 mmol) of $Zn(CH_3COO)_2 \cdot$ 2H2O in 187.5 ml of methanol in a 250 ml flask and heat to 60°C. Reaction mixture to be stirred for 10 minutes at this temperature.

- Dissolve 2.52 g (45 mmol) of KOH in 42.5 ml of methanol (35% less solvent then described in [(1)]) in another 100 ml flask and stir for 10 minutes at 60°C.
- Add the second solution to the first one during 30 sec. The solution becomes turbid and turns clear again after 5 min.
- Add reflux condenser and reflux the reaction mixture for 3 hours at 60°C.
- Use the rotary evaporator to remove most of the methanol.
- Cleaning: Add 80 ml of a 1:1 mixture of methanol and water. Sonicate for 10 min. and centrifuge for 15 min. Afterwards wash the particles three times with 80 ml of water.
- Freeze-thaw dry particles.

2.1.15.7 References

(1) WELLER et al., Angew. Chem. Int. Ed. 41 (2002), 1189 – 1191

2.1.16 SOP_2.36_Synthesis of UC-CU-Fe_2O_3 cubic NP_v1 - Solvothermal synthesis of cubic Fe_2O_3 nanoparticles

2.1.16.1 Purpose

To synthesize cubic Fe_2O_3 nanoparticles.

2.1.16.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of CU-Fe_2O_3 nanoparticles with ca. 44.3 nm diameter.

2.1.16.3 Principle

CU-Fe_2O_3 nanoparticles were synthesized with a solvothermal method.

2.1.16.4 Reagents and Materials

- $Fe(NO_3)_3 \cdot 9H_2O$
- PVP (Mw= 1.5 Mio)
- Dimethylformamid
- Ethanol
- Diionized water
- Autoclave
- Magnetic stirrers
- Centrifuge

2.1.16.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.1.16.6 Procedure

- 0.40g $Fe(NO_3)_3 \cdot 9H_2O$ (1 mmol) and 0.6g PVP were dissolved in 25 mL DMF, and the mixture was stirred at room temperature for 1 h.
- The final solution was transferred into a 50 mL autoclave, and was hold at 180°C for 30h.
- The resulting products were centrifuged three times at 10,500 rpm for 50min, washed 2 times by using ethanol, and washed 2 times by using diionized water.
- The obtained precipitate was dried under vacuum for 24 h.

2.2 Functionalisation and Coating

2.2.1 SOP Nanommune 2.13_v1 - Carboxylation of Single-Walled Carbon Nanotubes

2.2.1.1 Purpose

To oxidize single-walled carbon nanotubes for various studies where these nanoparticles can be applied in aqueous environments.

2.2.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the carboxylation of pristine single-walled carbon nanotubes.

2.2.1.3 Principle

Single-walled carbon nanotubes have excellent potential as drug delivery vehicles for biological applications. However, their hydrophobicity limits their solubility in aqueous systems. Partial oxidation to impart hydrophilicity remedies this issue.

2.2.1.4 Reagents and Materials

- 100 mL round bottomed flask
- 1 24/40 rubber septum
- 1 18G needle
- Disposable Pasteur pipettes
- Branson Bath Ultrasonicator Model 5510 (Frequency 40 kHz)
- Mass balance
- Thermometer
- Sample Vials (20 mL)
- Graduated Cylinder
- Teflon membrane filter (.22 µm)
- Analytical grade H_2SO_4
- 30% H_2O_2
- Pristine single-walled carbon nanotubes (P2, from Carbon Solutions)
- Analytical grade Ethanol
- High purity water

2.2.1.5 *Safety procedures and precautions*

- Standard safety operating procedures are to be followed at all times
- H_2SO_4/H_2O_2 (30%) is highly oxidizing. Caution must be taken when handling this solution
- All procedures (unless otherwise stated) are to be performed in a fume hood.
- Lab coats, latex gloves, and goggles must be worn at all times

2.2.1.6 *Procedure*

- Weigh out 20.0 mg of pristine single-walled carbon nanotube powder, and place in 100 mL round bottomed flask
- Measure 30 mL of H_2SO_4 and pour into 100 mL flask with nanotube powder
- Measure 10 mL of 30% H_2O_2 and add dropwise into the same round bottom flask. (Careful at this point as the solution will heat rapidly)
- Cap round bottom flask with a 24/40 septum, immediately following puncture by an 18 gauge needle as a pressure release
- Place the round bottom flask into the ultrasonicator bath.
- Change the bath temperature from ambient to 40°C. Verify with thermometer.
- Sonicate contents for 2 h.
- Carefully remove round bottomed flask from sonicator and remove and dispose of septum and needle.
- In a separate beaker (500 mL) dilute the contents of the round bottom flask 10× with pure distilled water
- Filter suspension over the Teflon membrane filter, washing with ethanol and additional water until neutral pH is achieved. Note that numerous membrane filters will be used
- Dry filters in ambient.
- Scrape product off with a spatula into a sample vial. Alternatively, nanoparticles can be sonicated off the filter into acetone. Evaporate acetone in ambient.

2.2.2 SOP Nanommune 2.14_v2 - Functionalization of Single-Walled Carbon Nanotubes with Fluorescein Isothiocyanate Fluorescence Labels

2.2.2.1 Purpose

To functionalize single-walled carbon nanotubes with fluorescein isothiocyanate (FITC) for various studies where these nanotubes can be applied for bioimaging using fluorescence microscopy.

2.2.2.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the functionalization of single-walled carbon nanotubes with FITC.

2.2.2.3 Principle

Single-walled carbon nanotubes have excellent potential as drug delivery vehicles for biological applications. FITC-functionalized SWNTs can be used for drug delivery study and bioimaging.

2.2.2.4 Reagents and Materials

- 100 mL round bottomed flask
- 25 mL round bottomed flask ×2
- Glass Vials (20 mL) ×3
- Graduated Cylinder
- 14/20 condenser ×2
- 14/20 septum ×4 4.7. 18G needle ×2
- Balloons
- Disposable Pasteur pipettes
- Branson Bath Ultrasonicator Model 5510 (Frequency 40 kHz)
- Labconco Rotary Evaporator
- Corning Hot Plate
- Fisher Scientific Centrifuge (Model 228)
- Mass balance
- Thermometer
- Teflon membrane filter (.22 μm)
- Pristine Single-Walled Carbon Nanotubes (P0329, From CNI)
- Analytical grade H_2SO_4
- Analytical grade HNO_3

- Analytical grade oxalyl chloride
- Analytical grade Boc-NH(CH_2CH_2O)2-$CH_2CH_2NH_2$
- Analytical grade THF
- Analytical grade methanol
- Analytical grade HCl
- Analytical grade p-dioxane
- Analytical grade diethyl ether
- Fluorescein isothiocyanate (FITC) purity >98%
- N,N-Diisopropylethylamine
- Analytical grade N,N-dimethylformide (DMF)
- High purity water

2.2.2.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- H_2SO_4/HNO_3 is highly oxidizing. Caution must be taken when handling this solution
- All procedures (unless otherwise stated) are to be performed in a fume hood.
- Lab coats, latex gloves, and goggles must be worn at all times

2.2.2.6 Procedure

- Weigh out 10.0 mg of pristine single-walled carbon nanotube powder, and place in 100 mL round bottomed flask
- Measure 15 mL of H_2SO_4 and pour into 100 mL flask with nanotube powder
- Measure 5 mL of HNO_3 and pour into 100 mL flask with nanotube powder.
- Place the round bottom flask into the ultrasonicator bath.
- Change the bath temperature from ambient to 40°C. Verify with thermometer.
- Sonicate contents for 3 h and 40 min. (Change the water in the bath at 1 hr, 2 hr, 3 hr with ambient tap water to avoid overheating)
- Carefully remove round bottomed flask from sonicator.
- In a separate beaker (500 mL) dilute the contents of the round bottom flask 10× with pure distilled water
- Filter suspension over the Teflon membrane filter, washing with ethanol and additional water until neutral pH is achieved. Note that numerous membrane filters will be used.
- Dry filters in ambient.

- Scrape product off with a spatula into a sample vial. Alternatively, nanoparticles can be sonicated off the filter into acetone. Evaporate acetone in ambient.
- Suspend 10 mg of oxidized SWNT in 4 ml of oxalyl chloride in a 14/20 25 mL round bottomed flask and put in a magnetic stirrer.
- Cap a 14/20 condenser on top of the 25 mL round bottomed flask.
- Cap condenser with a 14/20 septum, immediately following puncture by two 18 gauge needles as a pressure release
- Use a N_2 filled balloon for N_2 protection. Connect the balloon to one of the 18 gauge needles and leave the other needle for pressure release. Make sure the N_2 fill the system and then take off the needle for pressure release.
- Heat the round bottom flask in an oil bath on a hot plate and flux at the same time. Make sure the temperature of the oil bath is 62°C and keep the stirring on. Overnight.
- Stop the reaction and remove the excess of oxalyl chloride was evaporated under vacuum. (A)
- Suspend the product from (A) and 120 mg of Boc-NH(CH_2CH_2O)2-$CH_2CH_2NH_2$ in 5 ml of dry THF in a 14/20 25 mL round bottomed flask and put in a magnetic stirrer.
- Place a 14/20 condenser on top of the 25 mL round bottomed flask.
- Cap condenser with a 14/20 septum, following puncture by two 18 gauge needles as a pressure release
- Use a N_2 filled balloon for N_2 protection. Connect the balloon to one of the 18 gauge needles and leave the other needle for pressure release. Make sure the N_2 fill the system and then take off the needle for pressure release.
- Heat the round bottom flask in an oil bath on a hot plate and flux at the same time for 48 hr. Make sure the temperature of the oil bath is 62°C and keep the stirring on.
- Stop the reaction and cool to a room temperature.
- Remove THF under vacuum.
- Suspend the resulting SWNTs in methanol and centrifuge at 3000 rpm for 10 min. Remove the supernatant. Repeat four times. Then dry the precipitate under vacuum. (B)
- Suspend 5 mg of the products from (B) in 6 ml of 4 M HCl in dioxane in a 20 mL glass vial and put in a magnetic stirrer.
- Cap the vial with a 14/20 septum and fill in N_2.
- Stirring the mixture for 5 hr.
- Evaporate the solvent under vacuum.

- Wash the resulting SWNTs using diethyl ether using centrifugation for 4 times. (C)
- Suspend 5 mg of the products from (C), 2 mg of FITC and DIEA (catalyst) in 5 mL of dry DMF in a 20 ml glass vial.
- Cap the vial with a 14/20 septum and fill in N_2.
- Stirring the mixture for 5 hr.
- Filter suspension over the Teflon membrane filter, washing with ethanol and additional water until no FITC can be tested in the wash. Note that numerous membrane filters will be used.
- Dry filters in ambient.
- Scrape product off with a spatula into a sample vial.

2.2.2.7 References

(1.) J. Zhang, H. Zou, Q. Qing, Y. Yang, Q. Li, Z. Liu, X. Guo, Z. Du, Effect of Chemical Oxidation on the Structure of Single-Walled Carbon Nanotubes, J. Phys. Chem. B 2003, 107, 3712-3718

(2.) W. Wu, S. Wieckowski, G. Pastorin, M. Benincasa, C. Klumpp, J.-P. Briand, R. Gennaro, M. Prato, A. Bianco, Targeted Delivery of Amphotericin B to Cells by Using Functionalized Carbon Nanotubes, Angew.Chem. Int. Ed. 2005, 44, 6358.

(3.) V. K. Sarin, S. B. H. Kent, J. P. Tam, R. B. Merrifiled, Quantitative Monitoring of Solid-Phase Peptide Synthesis by the Ninhydrin Reaction, Anal. Biochem. 1981, 117, 147.

2.2.3 SOP_2.22 - Synthesis of Mandelic Acid modified ZnO nanoprisms_v1

2.2.3.1 Purpose

To synthesize nanoprismatic ZnO nanostructures for further mandelic acid modification. Synthesized and mandelic acid modified ZnO nanostructures were water soluble and redispersable.

2.2.3.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of ZnO nanoprisms.

2.2.3.3 Principle

Wurtzite ZnO structures were synthesized by thermolysis of Zn-oleate complex[1] .

Mandelic acid modification was developed at UCO.

2.2.3.4 Reagents and Materials

- Oleic acid
- Oleylamine
- Zn-oleate
- EtOH
- Hexane
- 250 ml three necked flask and various glassware
- Schlenk line and inert gas supply
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Mandelic acid
- Toluene
- MeOH

2.2.3.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous

- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times

2.2.3.6 *Procedure*

- Flask must be dry .
- Add oleic acid (5 eq) and Oleylamine (10 eq) into the Zn-oleate complex (1 eq). These conditions lead to nanoprism formation.
- Connect the refluxer and heating mantle and heat up to 290°C under Argon gas flow.
- Keep stirring the solution at this temperature for around 1 h.
- Reaction will be milky proving the formation of ZnO nanostructures.
- Let the mixture cool down to room temperature.
- Add required EtOH to the reaction mixture.
- Centrifuge the mixture and redisperse the nanostructures in hexane for removing the excess of any oleylamine and oleic acid reactants (3 times).
- Drying the nanostructures can be done either under nitrogen gas flow or vacuum.
- The following procedure was developed at UCO: Equal equivalent amounts of oleate capped ZnO and mandelic acid are dispersed in toluene and MeOH respectively.
- They are mixed and vigorous stirred for 5 minutes. Afterwards ultrasonic treatment is applied for 2 minutes.
- Solution is stirred for 16 hours. Solvents are removed by centrifugation and modified powders are washed with an acetone/hexane(3/1-v/v)-mixture.
- Particles were dried by a freeze-thaw-dry method developed at UCO: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.3.7 *Reference*

(1) S. CHOI, E. KIM, J. PARK, K. AN, N. LEE, S. KIM, T. HYEON, J Phys Chem B. 11 (2005) 14792-14794.

2.2.4 SOP_2.23 - Synthesis of Mandelic Acid modified ZnO nanoplates_v1

2.2.4.1 Purpose

To synthesize nanoplate ZnO nanostructures for further mandelic acid modification. Synthesized and mandelic acid modified ZnO nanostructures were water soluble and redispersable.

2.2.4.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of ZnO nanoplates.

2.2.4.3 Principle

Wurtzite ZnO structures were synthesized by thermolysis of Zn-oleate complex[(1)].

Mandelic acid modification was developed at UCO.

2.2.4.4 Reagents and Materials

- Oleic acid
- Oleylamine
- Zn-oleate
- EtOH
- Hexane
- 250 ml three necked flask and various glassware
- Schlenk line and inert gas supply
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Mandelic acid
- Toluene
- MeOH

2.2.4.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous

- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times

2.2.4.6 Procedure

- Flask must be dry .
- Add oleic acid (1 eq) and Oleylamine (5 eq) into the Zn-oleate complex (1 eq). These conditions leads to nanoplate formation.
- Connect the refluxer and heating mantle and heat up to 290°C under Argon gas flow.
- Keep stirring the solution at this temperature for around 1 h.
- Reaction will be milky proving the formation of ZnO nanostructures.
- Let the mixture cool down to room temperature.
- Add required EtOH to the reaction mixture.
- Centrifuge the mixture and redisperse the nanostructures in hexane for removing the excess of any oleylamine and oleic acid reactants (3 times).
- Drying the nanostructures can be done either under nitrogen gas flow or vacuum.
- The following procedure was developed at UCO: Equal equivalent amounts of oleate capped ZnO and mandelic acid are dispersed in toluene and MeOH respectively.
- They are mixed and vigorous stirred for 5 minutes. Afterwards ultrasonic treatment is applied for 2 minutes.
- Solution is stirred for 16 hours. Solvents are removed by centrifugation and modified powders are washed with an acetone/hexane(3/1-v/v)-mixture.
- Particles were dried by a freeze-thaw-dry method developed at UCO: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.4.7 Reference

(1) S. CHOI, E. KIM, J. PARK, K. AN, N. LEE, S. KIM, T. HYEON, J Phys Chem B. 11 (2005) 14792-14794.

2.2.5 SOP_2.24 Synthesis of Hydrothermal synthesis of UC-Fe_3O_4_40-1_v4 - Hydrothermal synthesis of ethylene glycol capped Fe_3O_4 nanoparticles.

2.2.5.1 Purpose

To synthesize ethylene glycol capped Fe_3O_4 nanoparticles.

2.2.5.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of Fe_3O_4 nanoparticles with ca. 56 nm diameter.

2.2.5.3 Principle

Fe_3O_4 nanoparticles were synthesized following a hydrothermal method [(1)].

2.2.5.4 Reagents and Materials

- $FeCl_3 \cdot 6H_2O$
- Ethylene glycol (EG)
- Sodium Acetate
- Ethanol
- Autoclave
- Magnetic stirrers
- Centrifuge

2.2.5.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.5.6 Procedure

- Adding $FeCl_3 \cdot 6H_2O$ (1 g, 6.2 mmol) and Sodium Acetate (3 g, 36.6 mmol) into a solution containing 20 ml ethanol and 10 ml PVP (40,000) in EG (0.6 M).
- After the mixture was stirred homogeneously, it was transferred into a 50 mL autoclave, and kept at 200°C for 4h.
- Precipitant (Fe_3O_4) was washed (3 times, mixture of water/EtOH) and collected by centrifugation.

2.2.5.7 Reference

(1) S. Guo, et al., Biomaterials 30 (2009), 1881.

2.2.6 SOP_2.25 Hydrothermal synthesis of UC-Fe_3O_4_50-1 _v4 - Hydrothermal synthesis of PVP capped Fe_3O_4 nanoparticles

2.2.6.1 Purpose

To synthesize PVP and ethylene glycol (EG) capped Fe_3O_4 nanoparticles.

2.2.6.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of Fe_3O_4 nanoparticles with around 38.5 nm diameter.

2.2.6.3 Principle

Fe_3O_4 nanoparticles were synthesized following a hydrothermal method [(1)].

2.2.6.4 Reagents and Materials

- $FeCl_3 \cdot 6H_2O$
- Ethylene glycol (EG)
- Sodium Acetate
- Ethanol
- Autoclave
- Magnetic stirrers
- Centrifuge

2.2.6.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.6.6 Procedure

- Add $FeCl_3 \cdot 6H_2O$ (1 g, 6.2 mmol) and Sodium Acetate (3 g, 36.6 mmol) into a solution containing 20 ml ethanol and 10 ml PVP in EG (0.6 M).
- After the mixture was stirred homogeneously, it was transferred into a 50 mL autoclave, and maintained at 200°C for 8h.
- Precipitant (Fe_3O_4) was washed (3 times, mixture of water/EtOH) and collected by centrifugation.

2.2.6.7 Reference

(1) S. Guo, et al., Biomaterials 30 (2009), 1881.

2.2.7 SOP_2.26 Hydrothermal synthesis of UC-Fe_3O_4_140-1 _v4 - Hydrothermal synthesis of PVP capped Fe_3O_4 nanospheres

2.2.7.1 Purpose

To synthesize PVP modified Fe_3O_4 nanospheres.

2.2.7.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of Fe_3O_4 nanospheres with ca. 127 nm diameter.

2.2.7.3 Principle

Fe_3O_4 nanospheres were synthesized following a hydrothermal method [1].

2.2.7.4 Reagents and Materials

- $Fe(NO_3)_3 \cdot 9H_2O$
- PVP-40.000
- NaOH
- DI water
- Autoclave
- Magnetic stirrers
- Centrifuge

2.2.7.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.7.6 Procedure

- Add $Fe(NO_3)_3$ (1 g), PVP (1 g) and NaOH (0.25 g) into 30 ml DI water.
- Mixture was transferred into a 50 ml autoclave, and kept at 200 oC for 8h.
- Precipitant (Fe_3O_4 nanospheres) were washed (3 times, mixture of water/ EtOH) and collected after centrifugation.

2.2.7.7 Reference

(1) S. GUO, et al., Biomaterials 30 (2009), 1881.

2.2.8 SOP_2.27 synthesis of nanocubes UC-Fe_2O_3_200-1 _v4 - Hydrothermal synthesis of PVP capped Fe_2O_3 nanocubes

2.2.8.1 Purpose

To synthesize PVP capped Fe_2O_3 nanocubes.

2.2.8.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of Fe_2O_3 nanocubes with around 156 nm diameter.

2.2.8.3 Principle

Fe_2O_3 nanocubes were synthesized following a hydrothermal method [(1)].

2.2.8.4 Reagents and Materials

- $Fe(NO_3)_3 \cdot 9H_2O$
- PVP-40.000
- NaOH
- $BaCl_2$
- Deionized water
- Autoclave
- Magnetic stirrers
- Centrifuge

2.2.8.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.8.6 Procedure

- Add $Fe(NO_3)_3$ (1.5 g, 6 mmol), $BaCl_2$ (0.6 g, 3 mmol), PVP (0.15 g, 4 µmol) and NaOH (0.75 g, 19 mmol) into 30 ml deionized water.
- Mixture was stirred for 30 min, and then transferred into a 50 ml autoclave, and keep at 200°C for 4h.
- Precipitant (Fe_2O_3 nanocubes) was washed (3 time, the mixture EtOH and water) and collected by centrifugation.

2.2.8.7 Reference

1) X, GUO, et al., J. Mater. Chem.19 (2009) 6706.

2.2.9 SOP 2.31 Synthesis of UC-ZnO-25-2_v1 - Synthesis of Mandelic acid Modified Zinc Oxide ENs

2.2.9.1.1 Purpose

To synthesize mandelic acid modified Zinc Oxide (ZnO) nanoparticles of ca. 12 ± 3 nm in diameter.

2.2.9.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of mandelic acid modified Zinc oxide nanoparticles.

2.2.9.1.3 Principle

Zinc oxide nanoparticles are synthesized by thermal decomposition of a zinc salt in high boiling solvent [1].

2.2.9.1.4 Reagents and Materials

- $ZnCl_2$
- NaOleate
- Oleic Acid
- Oleylamine
- Hexane
- Ethanol
- Deionized water
- DL Mandelic Acid
- Methanol
- Toluene
- 250 ml flask and various glass ware
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Ultrasonic bath

2.2.9.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.

- Treat all materials as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.9.1.6 Procedure

- First the zinc oleate precursor was synthesized by mixing 1.36 g (10 mmol) of zinc chloride with sodium oleate (6.08 g, 20 mmol) and adding hexane/ethanol/water mixture in 5/3/2 proportion according to their volumes, respectively. Reactants were refluxed for 4 hours at 70°C.
- Organic phase was extracted and aqueous phase was removed.
- Solvent mixture was removed by rotary evaporator and residual white product was dried under vacuum to obtain the precursor zinc oleate.
- For the synthesis of the ZnO nanoparticles, 1.54 g (1,7 mmol) Zn(Oleate)2 precursor was mixed with 19.3 ml (mmol) oleylamine and 9.7 ml (mmol) oleic acid in a three-necked flask. This mixture was heated with 5°C/min range using a thermocouple under an argon atmosphere until 285-290°C. Solution was kept for 1 h at this temperature.
- After 1 hour, the heater was removed and the mixture was brought to room temperature. Ethanol was added to precipitate the synthesized nanoparticles. Nanoparticles were washed 3 times with acetone and ethanol and dried under vacuum.
- For surface modification, 1 g (12.3 mmol) of dried ZnO particles were placed into a beaker and 50 ml toluene was added. This mixture was treated with an ultrasonicator for 30 minutes in order to get homogeneous suspension. 0.75 g (4.9 mmol) of mandelic acid was dissolved in 15 ml methanol separately in another beaker and added into the Toluene-ZnO mixture. This mixture was stirred at 65°C overnight.
- Freeze-thaw dry particles.

2.2.9.1.7 References

(1) Sang-Hyun Choi, Eung-Gyu Kim, Jongnam Park, Kwangjin An, Nohyun Lee,Sung Chul Kim, and Taeghwan Hyeon, J. Phys. Chem. B, Vol. 109, No. 31, 2005, 14792-14794

2.2.10 SOP 2.32 Microwave-assisted synthesis of UC-ZnO-80_v1 - Microwave-assisted synthesis of carboxylate capped ZnO

2.2.10.1.1 Purpose

To synthesize ZnO nanoparticles capped with carboxylic acid.

2.2.10.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of ZnO nanoparticles with ca. 86 nm diameter.

2.2.10.1.3 Principle

ZnO nanoparticles are synthesized with a microwave-assisted method [(1)].

2.2.10.1.4 Reagents and Materials

- $Zn(CH_3COO)_2{\cdot}2H_2O$
- Diethylene glycol (DEG)
- Microwave glass vessel
- Microwave reactor (CEM Discover-S Class)
- Magnetic stirrers
- Ethanol
- Deionized water
- Centrifuge

2.2.10.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.10.1.6 Procedure

- $Zn(CH_3COO)_2{\cdot}2H_2O$ (0.22 g, 1.0 mmol) was added into 40 mL diethylene glycol (DEG).
- The mixture was stirred at 70°C for 3h in order to get a homogeneous transparent solution. Then the solution (6 mL) was transferred into a 10 mL microwave glass vessel, and sealed with a Teflon cap.
- The reaction was hold at 200°C, for 15 min (300 W) under magnetic stirring.
- The product was collected by high speed centrifugation (11,000 rpm) for 15 min.

- The collected products were washed 3 times by a mixture of water and ethanol, and finally were dried under vacuum.

2.2.10.1.7 Reference

(1) X. Tang, E. Choo, L. Li, J. Ding and J. Xue., Langmuir 25(2009),5271.

2.2.11 SOP 2.33 Hydrothermal synthesis of UC-VC-Fe_3O_4_v - Hydrothermal synthesis of Vitamin C capped Fe_3O_4 nanoparticles.

2.2.11.1.1 Purpose

To synthesize Vitamin C (VC) capped Fe_3O_4 nanoparticles.

2.2.11.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of VC-Fe_3O_4 nanoparticles with ca. 3 nm diameter.

2.2.11.1.3 Principle

VC-Fe3O4 nanoparticles were synthesized with a hydrothermal method [(1)].

2.2.11.1.4 Reagents and Materials

- $FeCl_3 \cdot 6H_2O$
- Deionized water
- Sodium carbonate
- L-Ascorbic acid
- Autoclave
- Magnetic stirrers
- Centrifuge

2.2.11.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.11.1.6 Procedure

- 0.54 g $FeCl_3 \cdot 6H_2O$ (2 mmol) was dissolved in 25 mL deionized water, and the mixture was stirred at room temperature for 10 min.
- 10 mL, Na_2CO_3 water solution was introduced into the Fe^{3+} solution drop by drop. The color of the solution was changed to dark brown. The obtained solution was stirred at room for 30 min.
- After stirring for 30 min, proper amount of Vitamin C was added into this solution, and the mixture was further stirred for another 10 min.
- The final solution was transferred into a 50 mL autoclave, and was hold at proper temperature for 3h.

- The resulting products were centrifuged at 11,000 rpm for 1h, and washed 3 times by using deionized water.
- The obtained precipitate was dried under vacuum for 24 h.

2.2.11.1.7 Reference

(1) S. Guo, et al., Biomaterials 30 (2009), 1881.

2.2.12 SOP 2.34 Synthesis of UC-TiO_2-10_v - Synthesis of ethylene glycol capped Titanium Oxide Nanoparticles (EG@TiO_2)

2.2.12.1.1 Purpose

To synthesize ethylene glycol capped Titanium Oxide (EG@TiO_2) nanoparticles of ca. 7 nm diameter.

2.2.12.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of ethylene glycol capped titanium oxide (EG@TiO_2) nanoparticles.

2.2.12.1.3 Principle

Ethylene glycol capped titanium oxide (EG@TiO_2) nanoparticles were synthesized by sol-gel method followed by a thermal decomposition of a molecular precursor [(1)].

2.2.12.1.4 Reagents and Materials

- $Ti(OiPr)_4$
- Ethylene glycol
- H_2O
- Diethylether
- Ethanol
- 100 ml flask and various glass ware
- Magnetic stirrers
- Reflux condenser
- Oil bath
- Heating plate
- Thermocouple
- Centrifuge
- Ultrasonic bath
- Schlenk line and inert gas supply
- Liquid nitrogen

2.2.12.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.

- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.12.1.6 Procedure

- Flask must be dry and inert.
- Under vigorous stirring 0.25 ml $Ti(OiPr)_4$ was dropped into 37.5 ml ethylene glycol.
- The mixture was stirred until the white precipitation was dissolved again (about 30 minutes).
- 12.5 ml H_2O were added and the mixture was refluxed at 170°C for 2 hours.
- After cooling to room temperature, 37.5 ml of ethanol were added. 75 ml ether was added to precipitate the synthesized nanoparticles.
- Cleaning: Add 30 ml of ethanol and 60 ml ether. Sonicate the solution for 10 minutes and centrifuge for 15 minutes. The procedure should be repeated 3 times to remove residual surfactant.
- The particles were dispersed in H_2O, quick-freezed in liquid nitrogen and then freeze-dried at 0°C.

2.2.12.1.7 References

(1) Wang et al., J. Coll. Interf. Sci. 314(2007), 337 – 340

2.2.13 SOP_2.35_Synthesis of UC-GA-Fe_3O_4_v1 - Hydrothermal synthesis of gluconic acid capped Fe_3O_4 nanoparticles.

2.2.13.1.1 Purpose

To synthesize gluconic acid capped Fe_3O_4 nanoparticles.

2.2.13.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of GA-Fe_3O_4 nanoparticles with ca. 6 nm diameter.

2.2.13.1.3 Principle

GA-Fe_3O_4 nanoparticles were synthesized with a hydrothermal method [1].

2.2.13.1.4 Reagents and Materials

- 4.1. $FeCl_3 \cdot 6H_2O$
- 4.2. Deionized water
- 4.3. Sodium carbonate
- 4.4. Glucose
- 4.5. Autoclave
- 4.6. Magnetic stirrers
- 4.7. Centrifuge

2.2.13.1.5 Safety procedures and precautions

- 5.1. Standard safety operating procedures are to be followed at all times.
- 5.2. All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.13.1.6 Procedure

- 6.1. 0.54 g $FeCl_3 \cdot 6H_2O$ (2 mmol) was dissolved in 25 mL deionized water, and the mixture was stirred at room temperature for 10 min.
- 6.2. 10 mL, Na_2CO_3 water solution was introduced into the Fe^{3+} solution drop by drop. The color of the solution was changed to dark brown. The obtained solution was stirred at room for 30 min.
- 6.3. After stirring for 30 min, proper amount of glucose was added into this solution, and the mixture was further stirred for another 10 min.
- 6.4. The final solution was transferred into a 50 mL autoclave, and was hold at proper temperature for 3h.

- 6.5. The resulting products were centrifuged at 11,000 rpm for 1h, and washed 3 times by using deionized water.
- 6.6 The obtained precipitate was dried under vacuum for 24 h.

2.2.13.1.7 Reference

(1) X. Sun, C. Zheng, F. Zhang, Y. Yang, G. Wu, A. Yu and N. Guan., J. Phys. Chem. C (2009),113, 16002.

2.2.14 SOP_2.37_Synthesis of UC-GA-ZnO_v1 - Synthesis of gluconic acid modified ZnO spherical nanoparticles

2.2.14.1.1 Purpose

To synthesize spherical ZnO nanoparticles and applying phase transfer to modify them with gluconic acid. Synthesized gluconic acid modified ZnO spherical particles were water soluble and redispersable.

2.2.14.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of gluconic acid modified ZnO particles.

2.2.14.1.3 Principle

Wurtzite spherical ZnO particles were synthesized by sol-gel method from Zn-oleate(1) precursor. Phase transfer for gluconic acid modification was developed at UCO.

2.2.14.1.4 Reagents and Materials

- Zn-Oleate
- NaOH
- Water
- EtOH
- Hexane
- 250 ml three necked flask and various glassware
- Schlenk line and inert gas supply
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- % 50 water solution of gluconic acid
- Chloroform
- MeOH

2.2.14.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.

- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.14.1.6 Procedure

- Flask must be dry.
- Add 75 ml ETOH into 6.3 g Zn-oleate and dissolve it with ultrasonic treatment. Then heat this mixture up to 80°C. Prepare 0.6 g NaOH/75 ml MeOH mixture and add into the Zn-oleate at 80°C.
- Reflux the mixture at 80°C under nitrogen gas flow and water cooling around 72 hour.
- Let the mixture cool down to room temperature and add 150 ml hexane.
- Reaction will be milky during reflux proving the formation of ZnO nanostructures.
- Centrifuge the mixture and wash it with EtOH.
- Wash the mixture with hexane and water for removing the excess of any oleate (1-2 times) or water soluble groups.
- Drying the nanostructures can be done either under nitrogen gas flow or vacuum.
- After drying nanoparticles must be kept in dry conditions for preventing the humidity adsorption.
- The following procedure was developed at UC: 500 mg of ZnO NP and 300 mg gluconic acid are dispersed in chloroform and MeOH respectively.
- They are mixed and vigorous stirred for 5 minutes. Afterwards ultrasonic treatment is applied for 5 minutes for the phase transfer.
- Solution is stirred for 1 hour at 60°C. Solvents are removed by centrifugation and modified powders are washed with an EtOH, water and aceton respectively.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.14.1.7 Reference

1) S. CHOI, E. KIM, J. PARK, K. AN, N. LEE, S. KIM, T. HYEON, J Phys Chem B. 11 (2005) 14792-14794.

2.2.15 SOP_2.38_Synthesis of UC-CA-ZnO triangel NP_v1 - Synthesis of Citric Acid modified ZnO triangle shaped particles

2.2.15.1.1 Purpose

To synthesize triangle shaped ZnO nanostructures for and modify them with citric acid. Synthesized citric acid modified ZnO nanostructures were water soluble and redispersable.

2.2.15.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of citric acid modified ZnO triangle shaped particles.

2.2.15.1.3 Principle

Wurtzite ZnO structures were synthesized by thermolysis of Zn-oleate complex(1). Citric acid modification was developed at UC.

2.2.15.1.4 Reagents and Materials

- Oleic acid
- Oleylamine
- Zn-oleate
- EtOH
- Hexane
- 250 ml three necked flask and various glassware
- Schlenk line and inert gas supply
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Citric acid
- Toluene
- MeOH

2.2.15.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.

- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.15.1.6 Procedur:

- Flask must be dry.
- Add oleic acid (2.65 gr) and Oleylamine (11.82 g) into the Zn-oleate complex (5 g). These conditions leads to triangle shaped particle formation.
- Connect the refluxer and heating mantle and heat up to 300°C under nitrogen or Argon gas flow.
- When mixture reaches to 300°C, keep stirring the solution at this temperature for around 1 h.
- Reaction will be milky proving the formation of ZnO nanostructures.
- Let the mixture cool down to room temperature.
- Add 100 ml EtOH to the reaction mixture and disperse the particles.
- Centrifuge the mixture and redisperse the nanostructures in hexane for removing the excess of any oleylamine and oleic acid reactants (1-2 times).Then wash them with acetone with 2 times.
- Drying the nanostructures can be done either under nitrogen gas flow or vacuum.
- The following procedure was developed at UC: 500 mg of ZnO NP and 150 mg citric acid are dispersed in toluene and MeOH respectively.
- They are mixed and vigorous stirred for 5 minutes. Afterwards ultrasonic treatment is applied for 5 minutes.
- Solution is stirred for 16 hours at 70°C. Solvents are removed by centrifugation and modified powders are washed with an EtOH, water and aceton respectively.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.15.1.7 Reference

(1) S. CHOI, E. KIM, J. PARK, K. AN, N. LEE, S. KIM, T. HYEON, J Phys Chem B. 11 (2005) 14792-14794.

2.2.16 SOP_2.39_Synthesis of UC-FA-ZnO nanorods_v1 - Synthesis of Folic acid modified ZnO nanorods

2.2.16.1.1 Purpose

To synthesize nanorod shaped ZnO nanostructures and modify them with folic acid. Synthesized folic acid modified ZnO nanorods were water soluble and redispersable.

2.2.16.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of folic acid modified ZnO nanorods.

2.2.16.1.3 Principle

Wurtzite ZnO structures were synthesized by thermolysis of Zn-acetate dihydrate precursor [1]. Folic acid modification was developed at UC.

2.2.16.1.4 Reagents and Materials

- $Zn(acetate)_2.2H_2O$
- Oleylamine
- Water
- EtOH
- Hexane
- 250 ml three necked flask and various glassware
- Schlenk line and inert gas supply
- Magnetic stirrers
- Reflux condenser
- Heating mantle
- Thermocouple
- Centrifuge
- Folic acid
- Toluene
- MeOH

2.2.16.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.

- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.16.1.6 Procedure

- Flask must be dry.
- Add Oleylamine (5.07 g) into the Zn-acetate.dihydrate precursor (5 g). These conditions lead to ZnO nanorods.
- Connect the refluxer and heating mantle and heat up to 130°C under nitrogen or argon gas flow. Keep the mixture at this temperature around 45 minutes for degassing.
- Then heat the mixture again up to 250°C and keep at this temperature around 30 minute for nanorod formation.
- Reaction will be milky proving the formation of ZnO nanostructures.
- Let the mixture cool down to room temperature.
- Add 50 ml EtOH to the reaction mixture.
- Centrifuge the mixture and redisperse the nanostructures in hexane for removing the excess of any oleylamine (1-2 times).Then wash them with acetone with 2 times.
- Drying the nanostructures can be done either under nitrogen gas flow or vacuum.
- The following procedure was developed at UC: 500 mg of ZnO NP and 150 mg folic acid are dispersed in toluene and MeOH respectively.
- They are mixed and vigorous stirred for 5 minutes. Afterwards ultrasonic treatment is applied for 5 minutes.
- Solution is stirred for 16 hours at 70°C. Solvents are removed by centrifugation and modified powders are washed with an EtOH, water and aceton respectively.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.16.1.7 Reference

(1) Zhihua Zhang, Shuhua Liu, Shueyin Chow, and Ming-Yong Han, Langmuir (2006) 22, 6335-6340

2.2.17 SOP_2.40_Synthesis of UC-TA-TiO_2_v1 - Synthesis of Tartaric acid capped Titanium Oxide (TiO_2)

2.2.17.1.1 Purpose

To synthesize tartaric acid capped Titanium Oxide (TiO_2) nanoparticles soluble in water to check the influence of chiral molecules.

2.2.17.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of synthesized Titanium Oxide (TiO_2) nanoparticles.

2.2.17.1.3 Principle

Anatase Titanium Oxide nanoparticles are synthesized by a microwave procedure using a molecular precursor [(1)] with a following microwave treatment for the surface modification.

2.2.17.1.4 Reagents and Materials

- Isopropanol
- $Ti(OtBu)_4$
- DI water
- D-Tartaric acid
- L-Tartaric acid
- 15 ml beaker and various classware
- Magnetic stirrers
- Microwave
- Centrifuge

2.2.17.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.17.1.6 Procedure

- Add 0.3 ml (0.87 mmol) $Ti(OtBu)_4$ in 10 ml isopropanol.
- After stirring for 15 minutes at room temperature 0.6 ml (33 mmol) DI water are added dropwise.

- After rigorous stirring for 15 minutes fill each two Microwave tubes with 5 ml with the as prepared solution.
- Use the microwave with the following parameters: Fixed power mode under stirring, power 300 W, controlling temperature 250°C, controlling pressure 14.5 bar, time 15 minutes.
- After cooling to room temperature 60 mg (0.40 mmol) L- tartaric acid (or D-Tartaric acid) was added and mixed for 15 minutes.
- Use the microwave with the following parameters: Fixed power mode under stirring, power 300 W, controlling temperature 160°C, controlling pressure 14.5 bar, time 10 minutes.
- After cooling to room temperature remove the solvent using centrifugation.
- Washed the particles with a water/ethanol mixture (1:1) 3 times followed by a last washing procedure with 20 ml pure ethanol and dried overnight under atmospheric condition.

2.2.17.1.7 References

(1) Used as recieved from Acros Organics.

2.2.18 SOP_2.42_Synthesis of UC-SiO_2@Fe_2O_3 core/shell structure_v1 - Synthesis of aminopropyl SiO_2@ Fe_2O_3 core/shell modified Fe_2O_3 particles

2.2.18.1.1 Purpose

To synthesize aminopropyl SiO_2@Fe_2O_3 core/shell nanoparticles with two step method. Synthesized aminopropyl SiO_2@ Fe_2O_3 core/shell particles were water soluble and redispersable.

2.2.18.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of aminopropyl SiO_2@ Fe_2O_3 core/shell particles.

2.2.18.1.3 Principle

Amino propyl SiO_2@ Fe_2O_3 core/shell particles were synthesized by two step sol-gel method from the commercial nanoparticles. This method was developed at UC.

2.2.18.1.4 Reagents and Materials

- Commercial Fe_2O_3 nanoparticles (UC-bayferrox105M-1)
- conc. NH_3
- Water
- EtOH
- Acetone
- Tetraethoxysilane (TEOS)
- Aminopropyltriethoxysilane
- Magnetic stirrers
- 250 ml three necked flask and various glassware
- Ultrasonic bath
- Centrifuge tubes
- Centrifuge
- pH-meter

2.2.18.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.18.1.6 Procedure

- Flask must be dry.
- 500 mg Fe_2O_3 nanoparticle is dispersed as much as possible in 50 ml water.
- Prepare 30 ml water 100 ml EtOH mixture and add this into the Fe_2O_3 dispersion.
- Stir it around 10 minute.
- Add required amount of concentrated ammonia to bring the pH value up to around 10.0-10.5 which is measured by pH-meter.
- Stir this mixture around 30 minute.
- Add 2 ml TEOS (Tetraethoxysilane) very slowly. Whole addition must be completed in 30 minute-1 hour. Stir this mixture slowly around 20 hours at room temperature.
- Centrifuge the particles and wash them with EtOH and water 3 times. At this stage Fe_2O_3 nanoparticles are covered with a SiO_2 layer.
- Dry these particles at 80°C for 24 hours under vacuum.
- The following procedure was developed at UC: To increase the water solubility; 100 mg of SiO_2@ Fe_2O_3 structure was dispersed in 30 ml EtOH and stirred 30 minutes. Then 20 mg aminopropyl trimethoxysilane was dissolved in 10 ml EtOH/2 ml water mixture and added into the above solution. After stirring at 60°C around 10 hours, particles were centrifuged and washed with EtOH and aceton respectively.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.19 SOP_2.43_Synthesis of UC-SiO_2@ TiO_2 core/shell structure_v1Synthesis of amino propyl SiO_2@ TiO_2 core/shell modified TiO_2 particles

2.2.19.1.1 Purpose

To synthesize aminopropyl SiO2@TiO2 core/shell nanoparticles with two-step method. Synthesized aminopropyl SiO_2@ TiO_2 core/shell particles were water soluble and redispersable.

2.2.19.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of aminopropyl SiO_2@TiO_2 core/shell particles.

2.2.19.1.3 Principle

Amino propyl SiO_2@TiO2 core/shell particles were synthesized by two-step sol-gel method from the commercial nanoparticles. This method was developed at UC.

2.2.19.1.4 Reagents and Materials

- Commercial TiO2 nanoparticles (UC-p25-1)
- conc. NH3
- Water
- EtOH
- Acetone
- Tetraethoxysilane (TEOS)
- Aminopropyltriethoxysilane
- Magnetic stirrers
- 250 ml three necked flask and various glassware
- Ultrasonic bath
- Centrifuge tubes
- Centrifuge
- pH-meter

2.2.19.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.19.1.6 Procedure

- Flask must be dry.
- 500 mg TiO_2 nanoparticle is dispersed as much as possible in 50 ml water.
- Prepare 30 ml water 100 ml EtOH mixture and add this into the TiO_2 dispersion.
- Stirr it around 10 minute.
- Add required amount of concentrated ammonia to bring the pH value up to around 10.0-10.5 which is measured by pH-meter.
- Stir this mixture around 30 minute.
- Add 2 ml TEOS (Tetraethoxysilane) very slowly. Whole addition must be completed in 30 minute-1 hour. Stir this mixture slowly around 20 hours at room temperature.
- Centrifuge the particles and wash them with EtOH and water 3 times. At this stage TiO_2 nanoparticles are covered with a SiO_2 layer.
- Dry these particles at 80°C, 24 hours under or without vacuum.
- The following procedure was developed at UCO: To increase the water solubility; 100 mg of SiO_2@ TiO_2 structure was dispersed in 30 ml EtOH and stirred 30 minutes. Then 20 mg aminopropyl trimethoxysilane was dissolved in 10 ml EtOH/2 ml water mixture and added into the above solution. After stirring at 60°C around 10 hours, particles were centrifuged and washed with EtOH and aceton respectively.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.20 SOP_2.44_Synthesis of UC-SiO_2@ZnO core/shell structure_v1 - Synthesis of mercaptopropyl SiO_2@ZnO core/shell modified ZnO particles

2.2.20.1.1 Purpose

To synthesize mercapto propyl SiO_2@ZnO core/shell nanoparticles with two step method. Synthesized mercaptopropyl SiO_2@ZnO core/shell particles were water soluble and redispersable.

2.2.20.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of mercaptopropyl SiO_2@ZnO core/shell particles.

2.2.20.1.3 Principle

Mercapto propyl SiO_2@ZnO core/shell particles were synthesized by two-step sol-gel method from the commercial nanoparticles. This method was developed at UC.

2.2.20.1.4 Reagents and Materials

- Commercial ZnO nanoparticles (UC-zincox10-1)
- conc. NH_3
- Water
- EtOH
- Acetone
- Tetraethoxysilane (TEOS)
- Mercaptopropyl trimethoxysilane
- Magnetic stirrers
- 250 ml three necked flask and various glassware
- Ultrasonic bath
- Centrifuge tubes
- Centrifuge
- pH-meter

2.2.20.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.20.1.6 Procedure

- Flask must be dry.
- 500 mg ZnO nanoparticles are dispersed as much as possible in 50 ml water.
- Prepare 30 ml water 100 ml EtOH mixture and add this into the ZnO dispersion.
- Stir this mixture around 10 minute.
- Add required amount of concentrated ammonia to bring the pH value up to around 10.0-10.5 which is measured by pH-meter.
- Stir this mixture around 30 minute.
- Add 2 ml TEOS (Tetraethoxysilane) very slowly. Whole addition must be completed in 30 minute-1 hour period. Stir this mixture slowly around 20 hours at room temperature.
- Centrifuge the particles and wash them with EtOH and water 3 times. At this stage ZnO nanoparticles are covered with a SiO_2 layer.
- Dry these particles at 80°C for 24 hours under vacuum.
- The following procedure was developed at UC: To increase the water solubility; 100 mg of SiO_2@ZnO structure was dispersed in 30 ml EtOH and stirred 30 minutes. Then 20 mg mercaptopropyl trimethoxysilane was dissolved in 10 ml EtOH/2 ml water mixture and added into the above solution. After stirring at 60°C around 10 hours, particles were centrifuged and washed with EtOH and aceton respectively.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.21 SOP_2.45_Synthesis of UC-aminopropylsilane-TiO_2 v1 - Synthesis of aminopropyltriethoxysilane modified TiO_2 nanoparticles

2.2.21.1.1 Purpose

To synthesize aminopropyltriethoxysilane modified TiO_2 nanoparticles. Synthesized aminopropyltriethoxysilane modified TiO_2 particles were water soluble and redispersable.

2.2.21.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of aminopropyl triethoxysilane modified TiO_2 nanoparticles.

2.2.21.1.3 Principle

Aminopropyl triethoxysilane modified TiO_2 nanoparticles were synthesized by surface modification method from the commercial nanoparticles. This method was developed at UC.

2.2.21.1.4 Reagents and Materials

- Commercial TiO_2 particles (UC-p25-1)
- Aminopropyl triethoxysilane
- Water
- EtOH
- Acetone
- Heating oil bath
- Magnetic stirrers
- 250 ml three necked flask and various glassware
- Ultrasonic bath
- Centrifuge tubes
- Centrifuge

2.2.21.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.21.1.6 Procedure

- Flask must be dry.
- 500 mg TiO_2 nanoparticle are dispersed in 50 ml toluene.
- 150 mg aminopropyl triethoxysilane is dissolved in 10ml ethanol/1 ml water mixture.
- Two mixtures are mixed and sonicated around 5 minutes.
- This mixture is stirred at 70°C around 20 hours.
- Centrifuge the particles and wash them with EtOH and water 3 times and acetone at last.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.22 SOP_2.46_Synthesis of UC-mandelic acid-Fe_2O_3_v1 - Synthesis of mandelic acid modified Fe_2O_3 particles

2.2.22.1.1 Purpose

To synthesize mandelic acid modified Fe2O3 particles. Synthesized mandelic acid modified Fe_2O_3 particles were water soluble and redispersable.

2.2.22.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of mandelic acid modified Fe_2O_3 particles.

2.2.22.1.3 Principle

Mandelic acid modified Fe_2O_3 particles were synthesized by surface modification method from the commercial nanoparticles. This method was developed at UC.

2.2.22.1.4 Reagents and Materials

- Commercial Fe_2O_3 particles (UC-bayferrox105M-1)
- Mandelic acid
- Water
- EtOH
- Acetone
- Heating oil bath
- Magnetic stirrers
- 250 ml three necked flask and various glassware
- Ultrasonic bath
- Centrifuge tubes
- Centrifuge
- Toluene
- Methanol

2.2.22.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.22.1.6 Procedure

- Flask must be dry.

- 500 mg Fe_2O_3 nanoparticle are dispersed in 50 ml toluene.
- 100 mg mandelic acid is dissolved in 10 ml methanol.
- Two mixture are mixed and sonicated around 5 minutes.
- This mixture is stirred at 70°C around 20 hours.
- Centrifuge the particles and wash them with EtOH and water 3 times.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.2.23 SOP_2.47_Synthesis of UC-ZnO10-2_v1 - Synthesis of mandelic acid modified ZnO particles

2.2.23.1.1 Purpose

To synthesize mandelic acid modified ZnO particles. Synthesized mandelic acid modified ZnO particles were water soluble and redispersable.

2.2.23.1.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the synthesis of mandelic acid modified ZnO particles.

2.2.23.1.3 Principle

Mandelic acid modified ZnO particles were synthesized by surface modification method from the commercial nanoparticles.This method was developed chiocciola UC.

2.2.23.1.4 Reagents and Materials

- Commercial ZnO particles (UC-zincox10-1)
- Mandelic acid
- Water
- EtOH
- Acetone
- Heating oil bath
- Magnetic stirrers
- 250 ml three necked flask and various glassware
- Ultrasonic bath
- Centrifuge tubes
- Centrifuge
- Toluene
- Methanol

2.2.23.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times.

2.2.23.1.6 Procedure

- Flask must be dry.

- 500 mg ZnO nanoparticles are dispersed in 50 ml toluene.
- 300 mg mandelic acid is dissolved in 10 ml methanol.
- Two mixture are mixed and sonicated around 5 minutes.
- This mixture is stirred at 70°C around 20 hours.
- Centrifuge the particles and wash them with EtOH and water 3 times.
- Particles were dried by a freeze-thaw-dry method developed at UC: Particles were dispersed in water in a one neck flask. The suspension was frozen with liquid nitrogen. Subsequently the flask was set under high vacuum at a schlenk line for several hours until all solvent has been removed.

2.3 Material Characterisation

2.3.1 SOP Nanommune 2.04_v2 - TEM Characterization Techniques for Magnetite (Fe_3O_4) and Surface Coated Magnetite (Fe_3O_4) Nanoparticles

2.3.1.1 Purpose

The purpose of this SOP is to prepare uncoated and coated nanoparticles after synthesis for EM analysis.

2.3.1.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticles for TEM analysis

2.3.1.3 Principle

As nanoparticles are very small, transmission electron microscopy is required to study the interaction of nanoparticles with cells (e.g. uptake, localization). The TEM operates on the same principles as the light microscope but uses a beam of electrons instead of light. As the wavelength of electrons is much lower, it is possible to get a resolution a thousand times better than with a light microscope.

2.3.1.4 Reagents and Materials

- Disposable plastic pipettes
- Nanoparticles Sample
- Copper grid coated with formvar and carbon
- Ultrapure water (Elga Purelab Option)
- Scissors
- Tweezers

2.3.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.
- Indoor shoes must be worn.

2.3.1.6 Procedure

- From the original sample (stored in aqueous form), take 5 drops of the sample.
- Add the 5 drops into a glass vial (20mL) which contains 10mL of ultrapure water (Elgo Purelab Option).
- Sonicate (Sonics Vibra Cell) the sample for 1 minute
- The sample should be well mixed.
- Deposit one drop of nanoparticle suspension on a copper grid coated with formvar and carbon.
- Allow to dry for at least 24 h.

2.3.1.7 References

Transmission Electron Microscope: Theory of Transmission Electron Microscope (n.d.). Retrieved December 28, 2009 from http://em-outreach.ucsd.edu/web-course/toccontents.html.

2.3.2 SOP Nanommune 2.05_v2 - XRD Characterization Techniques for Magnetite (Fe_3O_4) and Surface Coated Magnetite (Fe_3O_4) Nanoparticles

2.3.2.1 Purpose

The purpose of this SOP is to prepare uncoated and coated nanoparticles after synthesis for XRD analysis.

2.3.2.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticles for XRD analysis

2.3.2.3 Principle

XRD system for phase analysis of polycrystalline samples is a versatile, non-destructive technique that reveals detailed information about the chemical composition and crystallographic structure of natural and manufactured materials [1].

2.3.2.4 Reagents and Materials

- Spoon
- Nanoparticles Sample
- Acetone
- Glass cover
- Scissors
- Tweezers

2.3.2.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

2.3.2.6 Procedure

- From the original sample (dry form), take at least 1mL of the sample.
- Place 1mL sample into a mortar and crush it until becomes a powder.
- Place the powder onto the amorphous sample holder.

- Uniformly disperse the powder onto the sample holder with the assistance of a glass cover.
- Place sample onto tool for analysis

2.3.2.7 References

1. X-Ray Diffraction: Theory of X-Ray Diffraction (n.d.). Retrieved December 28, 2009 from www.iucr.org/__data.

2.3.3 SOP Nanommune 2.6_v3 - DLS (Hydrodynamic Particle Sizer) Characterization Techniques for Magnetite (Fe_3O_4) and Surface Coated Magnetite (Fe_3O_4) Nanoparticles

2.3.3.1 Purpose

The purpose of this SOP is to prepare uncoated and coated nanoparticles after synthesis for particle size analysis.

2.3.3.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticles for DLS analysis

2.3.3.3 Principle

As nanoparticles are very small, hydrodynamic particle size analysis is required to study the interaction of nanoparticles with cells (e.g. uptake, localization). The principle of DLS utilizes a monochromatic and coherent laser light beam which illuminates a representative sample for analysis. The sample is dispersed at a desired concentration in a liquid. The light scattered by the particles at an angle (typically 90°) is recorded by a detector whose output is sent to a correlator. The decay of the autocorrelation function of the scattered intensity is interpreted in terms of average hydrodynamic particle size and polydispersity index, deemed as the cumulants method.

2.3.3.4 Reagents and Materials

- Disposable plastic pipettes
- Nanoparticles Sample
- Ethanol or Acetone
- Ultrapure water (Elga Purelab Option) or liquid of choice
- Glass or Disposable curvette

2.3.3.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times
- Indoor shoes must be worn

2.3.3.6 Procedure

- From the original sample (stored in aqueous form), take 5 drops of the sample.
- Add the 5 drops into a glass vial (20mL) which contains 10mL of ultrapure water (Elgo Purelab Option).
- Sonicate (Sonics Vibra Cell) the sample for 1 minute
- The sample should consist of well-dispersed particles in a liquid medium. (Note: the dispersion liquid should:
  - Be transparent (non-adsorbing) at the laser wavelength
  - Shall have a refractive index different from that of the particulate material
  - Compatible with the materials used in the instrument (Delsa Nano C)
  - Shall not dissolve, swell or coagulate the particle material
  - Shall be well filtered
- Deposit 2-3 drops of nanoparticle suspension into a curvette.
- With the pipette, mix the solution inside the curvette to ensure uniform mixture.
- Place curvette onto the tool (Delsa Nano C) and measure the intensity.
- If the intensity is acceptable, then proceed with the analysis and record the results.

2.3.3.7 References

(1) Dynamic Light Scattering: Theory of Dynamic Light Scattering (n.d.). Retrieved December 28, 2009 from http//:www.viscotek.com.

2.3.4 SOP Nanommune 2.07_v3 - TGA (Thermogravimetric Analysis) Characterization Techniques for Magnetite (Fe_3O_4) and Surface Coated Magnetite (Fe_3O_4) Nanoparticles

2.3.4.1 Purpose

The purpose of this SOP is to prepare uncoated and coated nanoparticles after synthesis for thermogravimetric analysis.

2.3.4.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticles for TG analysis.

2.3.4.3 Principle

The basic principle of TGA is employed as a characterization method that measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. This technique can provide the user with (1):

- Composition of multicomponent systems
- Thermal stability of materials
- Oxidative stability of materials
- Decomposition kinetics of materials
- Estimated lifetime of a product
- The effect of reactive or corrosive atmospheres on materials
- Moisture and volatile content of materials

2.3.4.4 Reagents and Materials

- Tweezers (Brass tweezers are recommended if available)
- Nanoparticles Sample
- Ethanol or Acetone
- TGA sample pan. Note: If your sample is in the liquid form use a hermeric pan with a pin-hole lid
- Spatula

2.3.4.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood.

- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times
- Indoor shoes must be worn.

2.3.4.6 Procedure

- From the original sample (stored in aqueous form), take 3-5 ml of the sample and dry (air or vacuum).
- Before the sample is run, place the cleaned pan onto the tool to be tared before every run.
- After the taring of the pan, evenly distribute the sample (5-15mg of dry sample is recommended) over the bottom of the pan.
- Place pan onto the tool (Q5000 IR) and measure the sample.
- After processing record and analyze the results.

2.3.4.7 References

(1) Thermogravimetric Analysis: Theory of Thermogravimetric Analysis (n.d). Retrieved December 28, 2009 from http://www.si-mex.com.mx/PDFS/orthon/Thermal%20Gravimetric%20Analysis%20brochure.pdf

2.3.5 SOP Nanommune 2.8_v2 - FTIR (Fourier Transform Infrared Spectroscopy) Characterization Techniques for Magnetite (Fe_3O_4) and Surface Coated Magnetite (Fe_3O_4) Nanoparticles

2.3.5.1 Purpose

The purpose of this SOP is to prepare uncoated and coated nanoparticles, after synthesis, for FTIR analysis.

2.3.5.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticles for FTIR analysis

2.3.5.3 Principle

FTIR spectroscopy is used primarily for qualitative and quantitative analysis of organic compounds, and also for determining the chemical structure of many inorganics. Because chemical bonds absorb infrared energy at specific frequencies (or wavelengths), the basic structure of compounds can be determined by the spectral locations of their IR absorptions. The plot of a compound's IR transmission vs. frequency is its "fingerprint", which when compared to reference spectra identifies the material (1).

2.3.5.4 Reagents and Materials

- Spatula
- Nanoparticles Sample
- Ethanol or Acetone
- Kemwipes

2.3.5.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.
- Indoor shoes must be worn.

2.3.5.6 Procedure

- From the original sample (stored in aqueous form), take 3-5 ml of the sample and dry (air or vacuum).

- Clean the sample holder, by acetone, with Kemwipes. Make sure not to splash the acetone on the instrument.
- Click the “back ground” button to collect back group information of the sample holder.
- Place you sample on the sample holder.
- If your sample is liquid, you can go ahead and press “Apply” and then “Start” to collect the spectrum.
- If your sample is solid, lower down the pressure arm. Press “Apply” and then “Start” to collect the spectrum.
- If the data is acceptable, then proceed with the analysis and record the results.

2.3.5.7 References

(1) PhotoMetrics, Inc. 15801 Graham St. , Huntington Beach CA 92649

2.3.6 SOP Nanommune 2.09_v2 - Zeta Potential Characterization Techniques for Magnetite (Fe_3O_4) and Surface Coated Magnetite (Fe_3O_4) Nanoparticles

2.3.6.1 Purpose

The purpose of this SOP is to prepare uncoated and coated nanoparticles after synthesis for isoelectric point analysis.

2.3.6.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticles for Zeta Potential analysis

2.3.6.3 Principle

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. A value of 25 mV (positive or negative) can be taken as the arbitrary value that separates low- charged surfaces from highly-charged surfaces. The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e. the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate(1).

2.3.6.4 Reagents and Materials

- Disposable plastic pipettes
- Nanoparticle(s) Sample
- Ethanol or Acetone
- Kemwipes
- Ultrapure water (Elga Purelab Option) or liquid of choice

2.3.6.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as hazardous.
- All procedures (unless otherwise stated) are to be performed in a fuming hood.

- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.
- Indoor shoes must be worn.

2.3.6.6 Procedure

- From the original sample (stored in aqueous form), take 3-5 ml of the sample and dry (air or vacuum).
- Load the cell - Simply insert the cell, close the lid and it's ready to go.
- Run the measurement - From the menu, select the standard operating procedure (SOP) you need or set your own conditions and click the 'start' button.
- If the data is acceptable, then proceed with the analysis and record the results.

2.3.6.7 References

(1) Zeta Potential: Theory of Zeta Potential (n.d.). Retrieved December 28,2009 from www.nbtc.cornell.edu/facilities/Zeta sizer.pdf

2.3.7 SOP_ 2.29_ions leaching_v1 - M_x+ ions leaching from the MO_x nanoparticle solution

2.3.7.1.1 Purpose

To study whether the metal ions are exist in the metal oxides solution (buffer and cell culture), and further study its effect on the toxicity.

2.3.7.1.2 Scope

This protocol is applicable to all members of the Nanommune project partener.

2.3.7.1.3 Principle

Metal oxide ion Leaching is from metal oxides buffer and cell culture solution. The concentrated solution can be dropped on silicon wafer, and the elemental composition of the final solid on the silicon wafer can be measured by EDX (Energy dispersive X-ray spectroscopy).

2.3.7.1.4 Reagents and Materials

- Metal oxides (ZnO, Fe_2O_3, Fe_3O_4, TiO_2)
- Buffer solution (Phosphate (Sodium) buffer, PH=7.4)
- Cell culture
- 25 ML one neck flask
- Magnetic stirrers
- Heating mantle
- Thermocouple
- Centrifuge
- Ultrasonic bath

2.3.7.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times
- Treat all material as hazardous
- All procedures (unless otherwise stated) are to be performed in a fuming hood. Lab coats, gloves and glasses must be worn at all times

2.3.7.1.6 Procedure

- 10 mg of the ZnO (or other MO_x NPs) was added into the 10 ml of the pH=7.4 phosphate buffer solution. (1 mg/1ml).
- Solution was sonicated for 5 minutes in order to get homogeneous dispersion.
- Obtained dispersion was stirred mildly and was heated in a water bath at 37°C for 24 h.

- After 24 h, the dispersion was centrifuged at 10,000 rpm for 15 minutes (repeat 2 times).
- Supernatant was seperated carefully from the nanoparticle precipitation and placed into a small sample bottle.
- Supernatant was evaporated at 105-110°C until around several droplets left.
- These droplets were transferred on to a Si wafer carefully.
- Si wafer samples were dried and prepared for the EDX measurement.
- The final solid on the silicon surface was measured by EDX.

2.4 Dispersion of ENs

2.4.1 SOP NANOMMUNE 3.07 v2 Dispersion of nanomaterials for in vitro experiments

(Partner 6: Tina Bürki)

2.4.1.1 Purpose

To obtain well-dispersed and reproducible suspensions of nanoparticles for biological testing with cultured cells.

2.4.1.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the dispersion of different nanomaterials for biological testing with primary cells and cell lines.

2.4.1.3 Background

Nanomaterials are often provided as powders and have to be suspended to obtain stable, homogenous dispersions that can be applied to the cultured cells. This can be difficult for some materials such as carbon nanotubes (CNTs), which are highly hydrophobic and tend to agglomerate. The degree and type of agglomeration are also known to influences the toxicity of the particles and thus might influence the interpretation of nanoparticle toxicity studies. Many different strategies have been used to disperse nanoparticles including high serum culture medium, surfactants, proteins, BALF or Arabic gum. Characterization of the resulting dispersions is essential to the interpretation of the toxicological data however; most of the methods used for material characterization are not compatible with high amounts of proteins or salts. Furthermore, the use of excessive amounts of amphiphilic molecules (e.g. proteins or serum components) should be avoided for some cell types such as macrophages as they blunt their response to particles. Thus, we will disperse the nanoparticles in aqueous suspensions whenever possible or add a non-ionic, biocompatible detergent such as Pluronic F127 if necessary. Pluronic does not interfere with the characterization of the suspensions and it has been shown that it is rapidly displaced by serum proteins thus under experimental conditions MWCNTs will be surrounded by serum components similar to the *in vivo* situation in the blood rather than a synthetic surfactant (Cherukuri et al. 2006). The protocol described here provides a first draft based on the knowledge of previous studies and experience in our labs. Therefore, modifications may be necessary for some specific nanomaterials that are not yet available or tested before or for other types of cells that require special treatment.

2.4.1.4 Preparation of stock suspensions

Stock suspensions of all nanomaterials should be prepared freshly if possible or 1 day before the experiments. Use new stock suspensions for each independent experiments.

- Carefully transfer the required amount of material to a clean glas tube
  → if possible use 1 mg/ml stock solutions; for CNTs use 250 µg/ml stock solutions (they tend to aggregate if conc. is too high)
- Add the appropriate suspension medium
  → use sterile, ultra pure water (LPS < 0.25 ng/ml) for hydrophilic particles
  → use ultra-pure water containing 160 ppm Pluronic F126 (Sigma P2443) for CNTs;
- Disperse the particles by sonication if required
  → for some particles a probe sonicator might be required (e.g. silica NPs, metal oxide NPs) whereas for others the use of an ultrasound waterbath is mandatory (e.g. CNTs where the rod would cause major defects in the lattice)
  → CNTs: sonicate for 10 min in an ultrasound bath (e.g. 600 W, Sonorex RK156 BH, Bandelin), vortex, sonicate again for 10 min; if supernatants are required (free of aggregates) spin the suspension for 10 min at 20'000g (Eppifuge), take supernatant and measure concentration of CNT using some of the stock suspension befor the centrifugation as a reference
  → silica NP: sonicate with a clean and sterile probe sonicator for 3x 20 sec (5 sec break) on ice
  → metal oxide NP: sonicate with a clean and sterile probe sonicator for 2 min on ice

2.4.1.5 Characterization of stock suspensions

- *Photo documentation*
  - Pictures of the stock suspensions may be carried out in a clear vessel in front of a suitable background.
- *Physicochemical characterization*
  - SEM or TEM micrographs should be analyzed to qualitatively characterize the size of particles in suspension.
  - Analytical ultra-centrifugation, laser diffraction or dynamic light scattering (e.g. Nanosight, advanced DLS) should be performed to quantitatively characterize the size of particles in suspension (can not be applied to high aspect-ratio particles such as CNTs).

- Zeta-potential of the stock suspensions should be measured to determine the surface charge of the particles
- If nanomaterials contain ions that can leach from the material, ICP-OES should be used to determine the amount of ions present in the stock suspensions (e.g. ZnO NPs)

- *Endotoxin contamination*
  - Endotoxins such as LPS would interfere with the toxicity testing[1]. Therefore stock suspensions will be tested for LPS contamination using the LAL test method (see SOP 3.08)

2.4.1.6 Use of stock suspensions for biological testing

For experiments, stock suspensions have to be serially diluted with the appropriate culture medium. Typical particle concentrations applied to the cells are in the range of 1-100 µg/ml. The time span from preparing the particle dilutions to the cell exposure should not exceed 30 min.

- Sonicate stock suspensions prior to the addition to the cell cultures if the time between preparing the stock and the dilution exceeds 30 min
  - CNTs: sonicate for 1 min in an ultrasound bath, carefully mix the tubes by turning the tube several times before pipetting the required amount to the culture medium. Use 10x concentrated medium to avoid dilution of the medium components. Always add 160 ppm Pluronic F127 to the medium (including the untreated controls) to avoid unspecific effects due to the surfactant.
  - Silica NP: sonicate with a clean and sterile probe sonicator for 3x 20 sec (5 sec break) on ice.

2.4.1.7 Characterization of experimental particle suspensions

2.4.1.7.1 Photo documentation

- Pictures of the experimental particle suspensions may be carried out in a clear vessel in front of a suitable background both at the beginning and the end of the experiments.

2.4.1.7.2 Physicochemical characterization

- Analytical ultra-centrifugation, laser diffraction or dynamic light scattering (e.g. Nanosight) should be performed to quantitatively characterize the size of particles in suspension (can not be applied to high aspect-ratio particles such as CNTs).
- Zeta-potential of the experimental particle suspensions should be measured to determine the surface charge of the particles

- If nanomaterials contain ions that can leach from the material, ICP-OES should be used to determine the amount of ions present in the culture medium.

2.4.1.8 *Safety procedures and precautions*

- Standard safety operating procedures are to be followed all times.
- Lab coates, nitril gloves or double vinyl or latex gloves must be worn all times
- When working with primary cells, treat them as infectious.

2.4.1.9 *References*

(1) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, et al. 2006. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. Nano letters 6(8): 1682-1686.

(2) Cherukuri P, Gannon CJ, Leeuw TK, Schmidt HK, Smalley RE, Curley SA, et al. 2006. Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence. Proceedings of the National Academy of Sciences of the United States of America 103(50): 18882-18886

3 In Vitro Toxicity Testing

3.1 Preparation of Cells and Cellular Subfractions

3.1.1 SOP NANOMMUNE 3.01v2 Preparation of human monocyte derived macrophages

(Partner 1a: Jingwen Shi)

3.1.1.1 Purpose

To prepare M-CSF-activated primary human macrophages from buffy coats for various studies.

3.1.1.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the isolation of peripheral blood mononuclear cells (PBMCs) using the Lymphoprep gradient centrifugation method, and the differentiation of monocytes into differentiated macrophages.

3.1.1.3 Principle

Whole blood is layered on top of Lymphoprep, a density gradient media, and the tube centrifuged, which results in the separation of the red blood cells, PBMCs and plasma. The red blood cells and granulocytes settle in the bottom of the tube and the PBMCs form a band above the Lymphoprep layer and the plasma rests on top of the PBMCs. Cells are removed from the tube and washed twice to remove the residues of Lymphoprep and platelets prior to plating in cell culture plates. To ensure optimal cell viability and recovery cells must be isolated within 24 h of preparation of the buffy coat.

3.1.1.4 Reagents and Materials

- 50 ml Falcon tubes (352070)
- Phosphate buffered saline pH 7.4
- Lymphoprep (Nycomed 1114545)
- Scissors
- Disposable plastic pipettes in 5 ml, 10 ml and 25 ml graduations
- Pipette aid
- Small autoclave bags
- RPMI 1640 medium (Sigma R0833) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Paisley, United Kingdom)

- RPMI medium (Sigma R0833) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Paisley, United Kingdom)
- 24-well plates (Falcon 353226)
- 0.5% Trypan blue solution
- Eppendorf tubes, 1.5 ml
- 70% denatured alcohol for disinfectant
- (Biohit Proline control decontaminating solution or similar)
- Recombinant human M-CSF (R&D systems, 216-MC)

3.1.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class II Safety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.1.1.6 Procedure

3.1.1.6.1 Preparation of Lymphoprep tubes

- Allow all solutions to reach room temperature
- Pipette 12.5 ml of lymphoprep into 4 x 50 ml Falcon tubes.

3.1.1.6.2 Centrifugation

- Cut one of the tubing of the buffy coat and aspirate 25 ml into 2 x 50 ml sterile Falcon tubes, add 25 ml PBS and mix by inverting tubes with the caps on carefully a few times
- Tilt tube and carefully pipette 25 ml of the diluted blood along the tube wall directly on top of the Lymphoprep.
- Spin at 1500 rpm for 25 min, without brake.
- Discard waste into double autoclave bags.

3.1.1.6.3 PBMC isolation

- Centrifugation results in tube contents dividing into four distinct layers:

    (1) Packed red cells, granulocytes at the bottom of the tube

    (2) Lymphoprep layer with (3) a white layer of PBMCs on top

    (4) Plasma

- Remove the plasma using a disposable pipette and a pipette aid or with vacuum suction into a special designated waste bottle, being careful not to disturb the PBMC layer beneath the plasma
- Carefully transfer the PBMC layer to a 50 ml Falcon tube (labeled HMDM) using a disposable pipette and pipette aid. Pool two fractions into one tube. Recap the remaining tube and discard in waste disposal bag.

3.1.1.6.4 PBMC wash and cell count

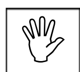
- Fill up with PBS to 50 ml and pellet cells by centrifugation for 5 minutes at 1200 rpm. Discard the supernatant and dislodge the pellet by gently tapping the tube against the palm of your hand. Resupend the cells in 10 ml PBS by aspirating the cell pellet up and down several times to break up any clumps. Resuspend to 50 ml using PBS, and mix well using a 25 ml pipette by drawing the sample up and down at least 3 times.
- Repeat one more time.
- Dilute PBMC fraction to approx. 30-50 ml by adding serum-free RPMI, and mix to homogeneity by inverting tube several times.
- Transfer 200 µl to an eppendorf tube, containing 800ul of PBS. Count cells in 3 A-squares in a Bürker chamber
- Record the results.

3.1.1.6.5 Selection of monocytes by plastic adherence

- Calculate the number of cells per ml.
- Dilute the PBMCs to 5·106 cells per ml in RPMI (w/o FBS).
- Add 1 ml of cell suspension per well in a 24-well plate and incubate at 37ºC for 1 h
- Wash out non-adhering cells at least two times with RPMI (w/o FBS).

3.1.1.6.6 Differentiation into activated macrophages

- After the final wash, add fresh complete RPMI medium supplemented with 50 ng/ml M-CSF.
- Culture cells for 3-4 days to generate fully differentiated and activated macrophages.



Note: Prior to exposure of macrophages to nanomaterials it is crucial to check the material for endotoxin contamination (e.g. lipopolysaccharide, LPS) (Vallhov et al., Nano Lett. 2006; Oostingh et al., Part Fibre Toxicol 2011). Endotoxins trigger immune responses in macrophages that may skew the readout in various assays.

3.1.1.7 References

(1) Oostingh G, Casals E, Italiani P, Colognato R, Stritzinger R, Ponti J, Pfaller T, Kohl Y, Ooms D, Favilli F, Leppens H, Lucchesi D, Rossi F, Nelissen I, Thielecke H, Puntes VF, Duschl A, Boraschi D. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Particle and Fibre Toxicology 2011; 8(1): 8.

(2) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, Gabrielsson S. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. Nano Lett. 2006; 6(8):1682-6.

(3) Manufacturer's protocol can be found at www.axis-shield-poc.com

3.1.2 SOP NANOMMUNE 3.5v1
Generation of human monocyte derived dendritic cells
(Partner 1b: Britta Andersson)

3.1.2.1 Purpose

To prepare human monocyte derived dendritic cells (MDDCs) from buffy coats for various studies.

3.1.2.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the isolation of peripheral blood mononuclear cells (PBMCs) from buffy coats, the separation of monocytes and their differentiation into MDDCs.

3.1.2.3 Principle

Blood from buffy coats is layered on the top of a Ficoll density gradient, and by centrifugation red blood cells, PBMCs and plasma become separated from each other. The red blood cells and granulocytes settle in the bottom of the tube and the PBMCs form a band above the Ficoll layer and the plasma rests on the top of the PBMCs. These are removed from the tube, washed and labelled with magnetic CD14 MicroBeads to receive the monocytes by a column separationMonocytes are then co-cultered with GM-CSF and IL-4 for 6 days to become MDDCs

3.1.2.4 Reagents and Materials

- 50 ml Falcon tubes (352070)
- Phosphate buffered saline pH 7.4
- RPMI 1640 medium (Sigma 30255.01), supplemented with 25 µg/mL gentamicin (Gibco Invitrogen Corporation, Paisley, UK), 2 mM L-glutamine (Gibco Invitrogen Corporation), 100 IU/ml penicillin (Gibco Invitrogen Corporation), 100 µg/ml streptomycin (Gibco Invitrogen Corporation), 50 µM β-mercaptoethanol (KEBO-lab, Spånga, Sweden), heat inactivated (56 ºC, 30 min) FCS (HyClone SH30071.03)
- Disposable plastic pipettes
- Pipette aid
- Scissors
- Trypan blue (Sigma-Aldrich T8154) and a Burker chamber
- Ficoll-Paque PLUS (GE Healthcare Bio-Science AB 17144003)

- CD14 MicroBeads, human (Miltenyi Biotec, 130050201) and autoMACS or separation columns (Miltenyi Biotec)
- Tissue culture flasks (Falcon, e.g. 353028)
- Conjugated mAbs specific for CD14, CD1a, CD11c, CD14 and CD83 and isotype controls (Becton Dickinson)
- IL-4 and GM-CSF (PHC0043 and PHC2013, Biosource International)
- EDTA
- BSA (Sigma, A1470-100G)

3.1.2.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class II Safety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.1.2.6 Procedure

3.1.2.6.1 Standard gradient centrifugation to receive human blood mononuclear cells from buffy coats

- Dilute the buffy coat with PBS 1:1 and then add 25 ml carefully to 15 ml Ficoll Paque at room temperature
- Centrifuge at 400 g without brake, 30 min, at room temperature.
- Collect upper phase if plasma is needed. Take the interface, which contains the PBMC, with a small pipette. Wash PBMC with PBS, spin at 300 g for 10 min. Pool and wash cells with PBS, spin at 200 g for 10 min.
- Count cells in Trypan blue in a Burker chamber. The viability should be around 90-100 %. Wash cells with PBS and spin at 200 g for 10 min.

3.1.2.6.1.1 Separation of monocytes (CD14^{+} cells)

- Resuspend cells in 80 µl ice-cold MACS buffer (PBS with 2 mM EDTA and 0.5 % BSA) per 10^{6} of total cells.
- Add 10 µl anti-CD14 beads for every 10^{6} cells. Resuspend carefully. Incubate for 30 min at 4 ºC.
- Separate CD14^{+} cells by autoMACS or columns according to manufacturer's instructions.

- Analyze 10 000 cells by flow cytometry to check for $CD14^+$ cell purity, by using conjugated mAbs specific for CD14 and its isotype controlThe sample should contain above 94 % $CD14^+$ cells.

3.1.2.6.2 Generation of monocyte derived dendritic cells

- Culture monocytes in RPMI 1640 medium, supplemented with 25 µg/mL gentamicin, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol, 10 %.heat inactivated fetal calf serum* and with the recombinant cytokines GM-CSF (550 IU/mL), and IL-4 (800 IU/mL), at a density of 4×10^5 cells/ml, in 37°C with a 6 % CO_2 atmosphere.
- After 3 days, remove 50 % of the medium by gentle pipetting. Add the same volume of fresh medium including new GM-CSF (1100 IU/mL) and IL-4 (1600 IU/mL) to the cells.
- After 6 days, analyze the cell surface molecules CD1a, CD11c, CD14 and CD83 by flow cytometry to confirm a phenotype of immature MDDC with high CD1a and CD11c expression (above 80 respectively 90 %), and low CD14 and CD83 expression (both below 10 %).[1, 2]

3.1.2.7 References

(1) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, Gabrielsson S. Mesoporous silica particles induce size dependent effects on human dendritic cells. Nano Lett. 2007; 7:3576-82.

(2) Romani N, Gruner S, Brang D, Kämpgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM, Schuler G. Proliferating dendritic cell progenitors in human blood. J. Exp. Med. 1994; 180:83-93.

* For generation of exosome free fetal calf serum, see SOP NANOMMUNE 3.3.

3.1.3 SOP NANOMMUNE 3.18 v2
Preparation of human monocyte derived macrophages using CD14 beads
(Partner 1a: Andrea Kunzmann)

3.1.3.1 Purpose

To prepare human monocyte derived macrophages (HMDM) from buffy coats for various studies.

3.1.3.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the isolation of peripheral blood mononuclear cells (PBMCs) from buffy coats, the separation of monocytes and their differentiation into HMDM.

3.1.3.3 Principle

Blood from buffy coats is layered on the top of a Lymphoprep density gradient, and by centrifugation red blood cells, PBMCs and plasma become separated from each other. The red blood cells and granulocytes settle in the bottom of the tube and the PBMCs form a band above the Lymphoprep layer and the plasma rests on the top of the PBMCs. These are removed from the tube, washed and labelled with magnetic CD14 MicroBeads to collect the monocytes by a column separationMonocytes are then co-cultered with M-CSF and for 3 days to become HMDM.

3.1.3.4 Reagents and Materials

- 50 ml Falcon tubes (352070)
- Phosphate buffered saline pH 7.4
- RPMI 1640 medium (Sigma 30255.01), supplemented with 25 µg/mL gentamicin (Gibco Invitrogen Corporation, Paisley, UK), 2 mM L-glutamine (Gibco Invitrogen Corporation), 100 IU/ml penicillin (Gibco Invitrogen Corporation), 100 µg/ml streptomycin (Gibco Invitrogen Corporation), heat inactivated (56 ºC, 30 min) FCS (HyClone SH30071.03)
- Disposable plastic pipettes
- Pipette aid
- Scissors
- Lymphoprep (Medinor, Sweden)
- CD14 MicroBeads, human (Miltenyi Biotec, 130050201) and autoMACS or separation columns (Miltenyi Biotec)

- Tissue culture plates (Falcon, 96-wells, 24-wells)
- M-CSF (Novakemi, Sweden)

3.1.3.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class IISafety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.1.3.6 Procedure

3.1.3.6.1 Standard gradient centrifugation to receive human blood mononuclear cells from buffy coats

- Dilute the buffy coat with PBS 1:1 (25ml blood + 25 ml PBS) and then add 25 ml carefully to 12.5 ml Lymphoprep at room temperature
- Centrifuge at 1500 rpm without brake, 30 min, at room temperature.
- Take the interface, which contains the PBMC, with a small pipette. Wash PBMC 2x with PBS, spin at 1200 rpm for 5 min.
- Resuspend the cells in 50ml PBS (one final tube for all cells), make a 1:10 dilution in an Eppendorf tube and count cells. Wash cells with PBS and spin at 1200 rpm for 5 min.

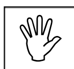
3.1.3.6.2 Separation of monocytes ($CD14^+$ cells)

- Resuspend cells in 80 µl ice-cold MACS buffer (PBS with 2 mM EDTA and 0.5 % BSA) per 10^7 of total cells.
- Add 10 µl anti-CD14 beads for every 10^7 cells. Resuspend carefully. Incubate for 15-30 min at 4 ºC.
- After incubation fill up to 50 ml with MACS buffer and spin at 1200 rpm for 5 min.
- Discard supernatant and resuspend $1x10^8$ cells in 500 µl MACS buffer
- Prepare magnetic separation by rinsing an LS column with 3 ml of MACS buffer
- Apply cell suspension to column and collect unlabeled cells in flow through (waste)
- Wash 3x by adding 3 ml of MACS buffer
- Place column on a 15 ml falcon tube outside of the magnetic field, add 5 ml buffer and use plunger to collect labelled cells
- Fill up cell suspension until 10 ml, make a 1:10 dilution in a Eppendor tube and count the cells. Spin cells at 1200 rpm for 5min.

- Resuspend cells in complete RPMI medium ($1x10^6$/ml) and seed them either on 96-well plates or 24-well plates (Falcon).

3.1.3.6.3 Generation of HMDM

- Culture monocytes in RPMI 1640 medium, supplemented with 25 µg/mL gentamicin, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 %.heat inactivated fetal calf serum* and with the recombinant cytokines M-CSF (50ng/mL), and at a density of $1x10^6$ cells/ml, in 37°C with a 6 % CO_2 atmosphere.
- Culture cells for 3-4 days to generate fully differentiated and activated macrophages.



Note: Prior to exposure of macrophages to nanomaterials it is crucial to check the material for endotoxin contamination (e.g. lipopolysaccharide, LPS) (Vallhov et al., Nano Lett. 2006; Oostingh et al., Part Fibre Toxicol 2011). Endotoxins trigger immune responses in macrophages that may skew the readout in various assays.

3.1.3.7 Reference

(1) Oostingh G, Casals E, Italiani P, Colognato R, Stritzinger R, Ponti J, Pfaller T, Kohl Y, Ooms D, Favilli F, Leppens H, Lucchesi D, Rossi F, Nelissen I, Thielecke H, Puntes VF, Duschl A, Boraschi D. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Particle and Fibre Toxicology 2011; 8(1): 8.

(2) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, Gabrielsson S. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. Nano Lett. 2006; 6(8):1682-6.

(3) Manufacturer's protocol can be found at www.axis-shield-poc.com

* For generation of exosome free fetal calf serum, see SOP NANOMMUNE 3.3.

3.1.4 SOP NANOMMUNE 3.3v1 Preparation of exosome free fetal calf serum (FCS)

(Partner 1b: Helen Vallhov)

3.1.4.1 Purpose

To prepare exosome free FCS.

3.1.4.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the preparation of exosomes free FCS using ultracentrifugation.

3.1.4.3 Principle

During high speed centrifugation the unwanted exosomes from FCS form a pellet which is discarded, while the supernatant is sterile filtered and saved for further preparation of exosome free cell media.

3.1.4.4 Reagents and Materials

- Ultra centrifuge tubes (Beckman Coulter 355655)
- RPMI 1640 medium (Sigma 30255.01)
- FCS (HyClone SH30071.03), heat inactivated (56 ºC, 30 min)
- Disposable plastic pipettes
- Pipette aid
- Filter, pores of.0.2 µm (Millipore, SCGVU05RE or 25CS020AS)

3.1.4.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class II Safety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.1.4.6 Procedure

- Centrifugation:
  Fill ultracentrifuge tubes with 30 % FCS and RPMI 1640 medium and centrifuge for 16-18 h at 100 000 g at 4 ºC without brake.
- Pour off the supernatant, which is the exosome free medium stock, and discard the pellet.

- Filtrate the supernatant through a filter and store at 4 ºC before further use in cell cultures.[1]

3.1.4.7 References

(1) Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, Raposo G, Amigorena S. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. J Cell Biol 1999; 147:599–610.

3.1.5 SOP NANOMMUNE 3.4v1
Protocol for isolating exosomes
(Partner 1b: Helen Vallhov)

3.1.5.1 Purpose
To isolate exosomes from cell culture supernatants for various studies.

3.1.5.2 Scope
This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the isolation of exosomes from cell media by ultracentrifugation.

3.1.5.3 Principle
Cell culture supernatants are first centrifuged at lower speeds to remove cells and debris. Thereafter, a higher speed is applied to receive the exosomes in a pellet, which protein content is measured by a protein assay.

3.1.5.4 Reagents and Materials
- Ultra centrifuge tubes (Beckman Coulter 355655)
- 50 ml Falcon tubes (352070)
- Phosphate buffered saline pH 7.4
- Disposable plastic pipettes
- Pipette aid
- Bio-RadDc protein assay (Bio-Rad 5000114)

3.1.5.5 Safety procedures and precautions
- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class IISafety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.1.5.6 Procedure
- Centrifugation

  Spin cell culture supernatants in a common cell centrifuge at 3 000 g for 20 min at room temperature. After this step the supernatants may be stored at –80ºC and are also transportable on dry ice.

- Ultracentrifuge supernatants at 10 000 g for 30 min at 4 ºC to eliminate remaining cells and debris (pellet).
- Centrifuge the supernatant at 100 000 g for 70 min at 4 ºC to receive the exosomes in the pellet. Retrieve the pellet by adding 200 µl of PBS and pipetting repeatedly. Fill the tube with PBS and repeat the last step for washing the pellet.
- Resupend the pellet in 50-200 µl PBS. If the exosomes are not used directly, store them at -80 ºC.
- Protein measurement
- Qauntitate the amount of the exosomal proteins by the Bio-RadDc protein assay based on the Lowry method according to the manufacturer's protocol. Store exosomes at -80 ºC.[1, 2]

3.1.5.7 References

(1) Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 1996; 183:1161–72.

(2) Johansson SM, Admyre C, Scheynius A and Gabrielsson S. Different types of in vitro generated human monocyte-derived dendritic cells release exosomes with distinct phenotypes. Immunology 2007:491-9.

3.2 Viability Assays

3.2.1 SOP NANOMMUNE 3.14v2 MTT cell viability assay

(Partner 1a: Andrea Kunzmann)

3.2.1.1 Purpose

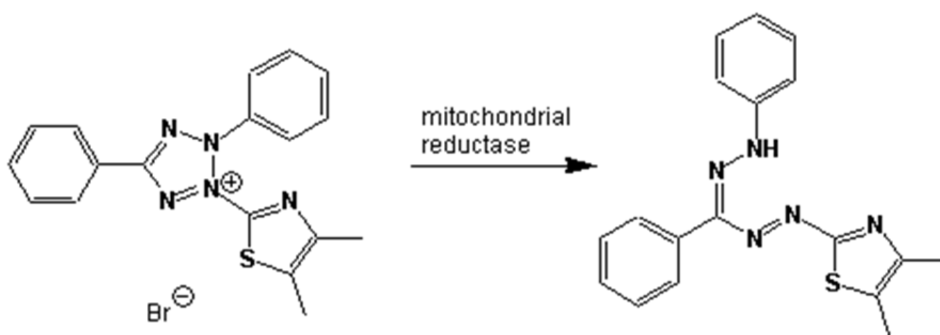
To test mitochondrial activity as a measure of cell viability after exposure to nanoparticles.

3.2.1.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection mitochondrial activity as a measure of cell viability after exposure to nanoparticles.

3.2.1.3 Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent and the released. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The absorption can be measured at 570 nm by a spectrophotometer.



The colorimetric reductions take place when reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells. However, it is important to keep in mind that other viability tests sometimes give different results, as many different conditions can increase or decrease metabolic activity. Changes in metabolic activity can give large changes in MTT results while the number of viable cells is constant. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control

cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve.

3.2.1.4 Reagents

- MTT Formazan (Sigma Aldrich, M2128) Synonyms:
- [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
- Methylthiazolyldiphenyl-tetrazolium bromide
- Phenolred free medium.
- DMSO.

3.2.1.5 Safety procedures and precautions

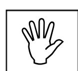
- Standard safety operation procedures are to be followed at all times.
- Avoid inhalation. Avoid contact with eyes, skin, and clothing
- Follow safety guidelines provided by the reagent and material manufacturer at all times.
- In contrast to older batches of MTT, which were declared as toxic, the new batches are declared as:.Not hazardous to human and the environment according to Directive 67/548/EC.
- Prevent contamination of the MTT solution, aseptically remove the appropriate volume for use during the entire experiment and place it into a separate tube. Return the remainder to storage at 2 - 8° C in the dark. Contamination will compromise the stability of this reagent.

3.2.1.6 Procedure

- Plate 200µl of $5x10^4$ cells /ml (cell line) or $1x10^6$ cells /ml (primary cells) in a 96-well plate for 24h (cell line) or until generation of requested cell type. Preferably plate triplicates of each sample.
- Expose cells to nanoparticles, negative controls and positive controls.
- If needed take along a cell free sample, adding all reagents during the protocol except for cells.
- Wash cells with 200µl PBS
- Add 100µl MTT/well (0.5mg/ml in phenolred-free complete medium)
- Incubate cells for 3-4 h at 37°C. View the cells periodically for the appearance of punctate, intracellular precipitate using an inverted microscope. Longer incubation times (up to 24 hours) may be required, depending on the cell type and experimental conditions. When purple precipitate is clearly visible under the microscope, add 50 µl of DMSO to all wells.
- Remove media (in special nanomaterial waste bottle) and add 50µl DMSO/well.

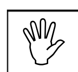
- Leave plate covered in the dark. Shake the plate very gently by hand. Usually the plates can be measured after several minutes.
- Measure the absorption at 570nm
- Determine the average values from triplicate readings and subtract the average value for the blank. Plot absorbance on the y-axis versus treatment on the x-axis. Calculate cell viability by taking the negative control samples as 100%.

3.2.1.7 Control for assay-nanoparticle interactions



There are several publications pointing to limitations in using the MTT assay to test toxicity of nanoparticles (Oostingh et al., Part Fibre Toxicol 2011). Monteiro-Riviere et al. (2009) observed that carbon based nanomaterial caused a false-positive reaction by interacting with the dye. In addition, Laaksonen et al. (2007) reported that the MTT assay may yield erroneous information with regard to the toxicity of porous silicon microparticles due to interference of the silicon particles with the tetrazolium salt, MTT. To investigate possible interference, examine the absorbance of MTT solution incubated with cell free suspension of nanomaterials at varius concentrations and compare the absorbance to a control solution of the dye

3.2.1.8 Procedure



- Prepare 100 µl of desired concentration of nanoparticles in MTT working solution (0.5mg/ml in phenolred-free complete medium) and negative controls (MTT working solution alone). The experiment should be done in triplicates.
- Incubate for 3 h at.37°C.in the dark.
- Measure the absorption at 570nm.
- Determine the average values from triplicate readings and compare whether there is differences in absorption between control and nanoparticles samples. If the nanomaterials interfere with the assay another method to evaluate cell viability should be considered.

3.2.1.9 References

(1) Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys. 1993;303(2):474-82.

(2) Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. 1991;3(7):207-12.

(3) Laaksonen T, Santos H, Vihola H, Salonen J, Riikonen J, Heikkilä T, Peltonen L, Kumar N, Murzin DY, Lehto VP, Hirvonen J. Failure of MTT as a toxicity testing agent for mesoporous silicon microparticles. Chem Res Toxicol. 2007; 20(12):1913-8.
(4) Monteiro-Riviere N.A., Inman A.O. Zhang L.W. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol Appl Pharmacol 2009; 243:222-235.
(5) Oostingh G, Casals E, Italiani P, Colognato R, Stritzinger R, Ponti J, Pfaller T, Kohl Y, Ooms D, Favilli F, Leppens H, Lucchesi D, Rossi F, Nelissen I, Thielecke H, Puntes VF, Duschl A, Boraschi D. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Particle and Fibre Toxicology 2011; 8(1): 8.

3.2.2 SOP NANOMMUNE 3.29 MTT cell viability assay

(Partner 10a: Nancy A. Monteiro-Riviere)

3.2.2.1 Purpose

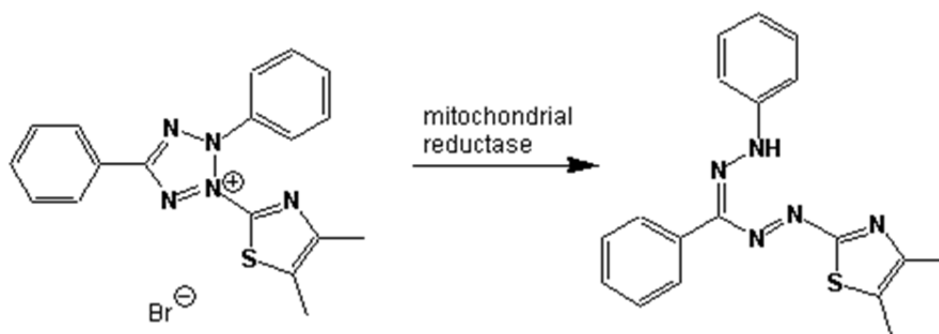
Use of mitochondrial activity to measure the viability of cells following timed exposure to nanoparticles.

3.2.2.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the measure of mitochondrial activity to quantify cell viability following exposure to nanoparticles.

3.2.2.3 Principle

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble dark purple formazan product. The formazan product within the cells is then solubilized and the absorption measured at 570 nm by a spectrophotometer.



In theory, the reduction of MTT can only occur in metabolically active cells with the level of activity a measure of the viability of the cells. The colorimetric reduction takes place when reductase enzymes are active, and therefore conversion is often used as a measure of viable (living) cells. However, it is important to keep in mind that other viability tests sometimes give different results, as many different conditions can increase or decrease metabolic activity. Changes in metabolic activity can give large changes in MTT results while the number of viable cells is

constant. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve.

3.2.2.4 Reagents

- MTT Formazan (Sigma Aldrich, M2128) Synonyms:
- - [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
- - Methylthiazolyldiphenyl-tetrazolium bromide
- Cell culture medium.
- 70% isopropyl alcohol (IPA)

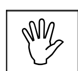
3.2.2.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times. Avoid inhalation and contact with eyes, skin, and clothing. Follow safety guidelines provided by the reagent and material manufacturer at all times.
- In contrast to older batches of MTT, which were declared as toxic, the new batches are declared as: Not hazardous to human and the environment according to Directive 67/548/EC.
- Prevent contamination of the MTT reagent. Aseptically remove and weigh the appropriate mass for use during the entire experiment and place it into a separate sterile tube. Return the remainder to storage at 2 - 8° C in the dark. Contamination will compromise the stability of this reagent.

3.2.2.6 Procedure

- Plate cells at appropriate concentration in 200µl of medium in the inner wells of a 96-well plate for 24h. The outer wells of the plate are filled with media or Hanks' Balanced Salt Solution (HBSS) to help minimize evaporation of the medium on the cells.
- Expose cells to nanoparticles, negative controls, and positive controls relevant to the treatment protocol at 37°C under cell culture conditions.
- Carefully aspirate treatment medium, pool by treatment, aliquot, and store at -80°C for later cytokine analysis. Add 200µl pre-warmed MTT medium (0.5mg/ml in complete culture medium) to each well and incubate cells for 3h at 37°C under cell culture conditions.

- Remove the plates from the incubator, carefully aspirate the MTT medium, and add 200µl HBSS to the cells for 2 minutes to rinse. Carefully aspirate HBSS and add 100µl of 70% IPA to the treatment wells to desorb formazan from the cells.
- Place the plate on a plate shaker at 300rpm for at least 20 minutes. The desorbed formazan in 70% IPA is transferred to a new 96-well plate to avoid interference with the nanoparticles attached to the cells in the bottom of the wells and the absorbance read at 540nm.
- Calculate the mean values from multiple wells and normalize to the control (untreated) cells to report as percent cell viability.
- Determine possible interferences of the dye with the nanoparticles.



Nanoparticle control: Coat wells in 96-well plate with rat tail collagen to help provide adhesion of nanoparticles to the bottom of the wells. Dose wells with nanoparticle concentrations used in viability studies and incubate plates for 24h under cell culture conditions (to mimic viability studies). Centrifuge the plates on the same setting as cells to compact the nanoparticles, carefully aspirate media (leaving nanoparticles in the wells), and add 200µl MTT medium to the wells for 3h. Centrifuge plate, carefully aspirate media (leaving nanoparticles in the wells), rinse with HBSS, and resuspend nanoparticles in 70% IPA. Agitate and read absorbance at 540nm. Any change from control (well with no nanoparticles) indicates nanoparticle interference with the formazan dye.

Cell control: Incubate plate containing nanoparticles (as above) in tandem with plate containing cells cultured at appropriate concentration. After 24h, the normal cells are assayed with MTT for 3h and the absorbance quantitated. The plate is centrifuged, the media removed from the wells in the plate containing the nanoparticles, and the MTT media is transferred to the wells containing the nanoparticles. The plate is incubated for 1h, centrifuged, the media transferred to wells in a new plate, and the absorbance quantitated. Any difference between the absorbance from the cells and the absorbance in the nanoparticles indicates nanoparticle interference.

✋: false-positive or false-negative results [2-8]

3.2.2.7 *References*

(1) Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 65, 55-63.

(2) Belyanskaya, L., Manser, P., Spohn, P., Bruinink, A., and Wick, P. (2007). The reliability and limits of the MTT reduction assay for carbon nanotubes-cell interaction. Carbon 45, 2643-2648.

(3) Monteiro-Riviere, N. A. and Inman, A. O. (2006). Challenges for assessing carbon nanomaterial toxicity to the skin. Carbon 44, 1070-1078.

(4) Casey, A., Herzog, E., Davoren, M., Lyng, F. M., Byrne, H. J., and Chambers, G. (2007). Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity. Carbon 45, 1425-1432.

(5) Wörle-Knirsch, J. M., Pulskamp, K., and Krug, H. F. (2006). Oops they did it again! Carbon nanotubes hoax scientists in viability assays. Nano Lett. 6, 1261-1268.

(6) Monteiro-Riviere NA, Inman AO, Zhang LW. (2009). Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicology and Applied Pharmacology. 234, 222-235.

(7) Samberg ME, Oldenburg SJ, Monteiro-Riviere NA. Evaluation of silver nanoparticle toxicity in vivo skin and in vitro keratinocytes. Environmental Health Perspectives 118, 407-413, 2010.

(8) Monteiro-Riviere NA, Oldenburg SJ, Inman AO. Interactions of aluminum nanoparticles with human epidermal keratinocytes. Journal of Applied.Toxicology 30:276-285, 2010.

3.2.3 SOP NANOMMUNE 3.6 v1 Detection of apoptosis by FACS: Annexin V/PI staining

(Partner 6: Tina Bürki)

3.2.3.1 Purpose

To detect apoptotic and late apoptotic/necrotic cells after exposure to nanoparticles.

3.2.3.2 Scope

This protocol is applicable to all members of the Nanommune project and provides a descriptive procedure detailing the detection of apoptotic and late apoptotic/necrotic cells after exposure to nanoparticles by flow cytometry.

3.2.3.3 Background/Principle

Background: Apoptosis is a fundamental mode of cell death which performs a regulatory function during normal development, in tissue homeostasis, and in some processes. In normal viable cells phosphatidyl serine (PS) is located in the inner leaflet of the plasma membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids in most cell types occurs leading to exposure of PS on the cell surface. Recognition of PS by phagocytes *in vivo* results in the removal of cells programmed to die thus apoptosis is not commonly associated with the local inflammatory response which accompanies necrosis.

In vitro detection of externalized PS can be achieved through interaction with the anti-coagulant annexin V (AV). In the presence of calcium, rapid high affinity binding of annexin V to PS occurs. PS translocation to the cell surface precedes nuclear condensation, DNA fragmentation, and the appearance of most apoptosis-associated molecules making annexin V binding a marker of early-stage apoptosis.

Principle: In this assay a fluorescein isothiocyanate (FITC) conjugate of annexin V is used allowing detection of apoptosis by flow cytometry. Since membrane permeabilization is observed in necrosis, necrotic cells will also bind annexin V-FITC. Propidium iodide (PI) is used to distinguish between viable, early apoptotic, and necrotic or late apoptotic cells. Due to its size and hydrophilic properties PI can not pass the intact membrane. Necrotic cells will bind annexin V-FITC and stain with PI while PI will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. Final stages of apoptosis involve necrotic-like disintegration of the total cell, thus cells in late apoptosis will be labelled with both FITC and PI.

Controls:

- Staurosporine is a potential inhibitor of protein kinase C and cells exposed to staurosporine will undergo necrosis.
- The Fas-activating ab and Fas Ligand induces the trimerization of the Fas-receptors, thereby activating apoptosis.

3.2.3.4 Reagents

- Polystyrene FACS tubes (BD Biosciences, 352052)
- FITC Annexin V (BD Biosciences, 556419)
- Propidium Iodine Staining solution (BD Biosciences, 556463)
- Binding Buffer
- 10 x Annexin V Binding Buffer (BD Biosciences, 556454) or
- 10 x Binding buffer (100mM Hepes pH 7.4, 1.5M NaCl, 50mM KCl, 10 mM $MgCl_2$, 18 mM $CaCl_2$):
- 2.38g Hepes
- 8.766g NaCl
- 0.373g KCl

*3.2.3.5 0.433g $MgCl_2$*$6H_2O$*

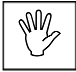
- 0.350g $CaCl_2$*$2H_2O$
- Dissolve in 100 ml dH_2O and adjust to pH 7.4 with NaOH
- 1x PBS (176.8 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4,8.1 mM Na_2HPO_4 x $2H_2O$)
- Staurosporine (Antibiotic AM-2282) (Produkt Nr. S 5921, Sigma). 1 Unit of 0.5 mg, MW: 466.5. Staurosporine is diluted in DMSO (1 mM; 500 µg in 1072 µl DMSO), aliquoted and stored at– 20° C
- Fas-activating antibody (human activating, mouse IgM) (Product Nr. 05-201, clone CH-11, Upstate, Temecula, CA). Formulation: 500µg/ml in PBS, 50% glycerol. The antibody is diluted in media before use: working stock: 10µg/ml; final concentration: 100ng/ml
- Fas Ligand (SuperFasLigand, Soluble (human) (recombinant); Product Nr. ALX-522-020, Alexis Biochemicals)

3.2.3.6 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times

- Propidium iodide is a potential carcinogen. Wear suitable protective clothing, gloves and eye protection.

3.2.3.7 Procedure



Incubate the cells with different concentrations of nanoparticles for the desired time
Suggested controls: Staurosporine (necrosis), Fas antibody or Fas Ligand (apoptosis)

- Collect the cells in a polystyrene FACS tube
- Wash cells with cold PBS
- Count cells and transfer 1x 10^5 cells to a centrifugation tube
- Centrifuge and resuspend cells in 100 µl 1x Binding Buffer
- Add 5 µl of FITC Annexin V
- Add 5-10 µl of PI (the optimal concentration of PI may vary among cell lines)
- Gently vortex the cells and incubate for 15 min at room temperature in the dark
- Add 400 µl of 1x Binding Buffer to each tube and analyze by flow cytometry within 1 hr

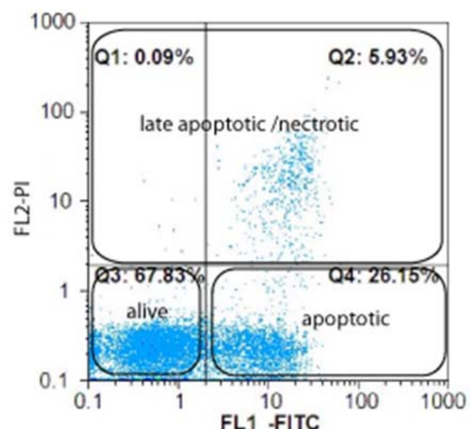
Suggested controls for setting up flow cytometry (compensation and quadrants):

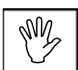
- Unstained cells
- Cells stained with FITC Annexin V (no PI)
- Cells stained with PI (no FITC Annexin V)
- Untreated cells stained with PI and FITC Annexin V

Controls 1-3 are best performed with cells that were induced to undergo apoptosis (e.g. by Fas antibody or Fas Ligand)Untreated cells are required to set the quadrants.

AV- FITC: Ex: 488 nm Em: 518 nm
PI: Ex: 488-540 nm Em: 617 nm

Alive: AV-/ PI-
Apoptotic: AV+ /PI-
Late apoptotic/necrotic: AV- / PI+ and AV+ /PI+

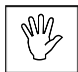




Note: Before introducing nanomaterials into the assay to investigate potential effects on apoptosis, it is important to include controls to check for potential interference with this assay.

However, be aware that there is no perfect control for the recognition/substraction of nanoparticle specific effects.

Suggested control experiments:



Include one sample adding the highest concentration of nanomaterials used for the assay but without cells. This sample is treated equally to all the other experimental samples, and FACS measurement is performed for approximately the same time as it takes for 10’000 cells. This control will give a feeling if nanoparticle aggregates/agglomerates appear in the scatter plot and if these are stained with either PI or AV. However, it will not always be possible to set a gate to remove these interfering particles in the experimental measurements as the particles might co-locate with the cell cloud (in this case a gate would also remove some of the real signals) or due to the fact that in an experimental sample, the particles might stick to the surface of cells and behave different than in this particle-only control (in this case no gate can be set as the aggregates will move to a different position in the scatter plot due to their association with the cells)!

In our experience, it is sometimes possible to reasonably gate out most of the nanoparticle unspecific signals. However, if this is not possible, the analysis often still can be performed up to a certain concentration (often up to 30-50 µg/ml nanoparticles) before nanoparticle-interference starts to really affect the outcome of the test.

3.2.3.8 References

(1) Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods . 1995; 184(1): 39-51.

(2) van Engeland M, Ramaekers FC, Schutte B, Reutelingsperger DP. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry. 1996; 24(2): 131-139.

(3) Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994; 84(5): 1415-1420.

3.2.4 SOP NANOMMUNE 3.12 v.2 CytoTox 96 Non-Radioactive Cytotoxicity Assay

(Partner 1a: Britta Andersson)

3.2.4.1 Purpose

To detect cytotoxicity (cell membrane damage) after exposure to nanoparticles.

3.2.4.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection of cytotoxicity (cell membrane damage) after exposure to nanoparticles.

3.2.4.3 Background/Principle

Background: Exposure of cells to various toxic substances can lead to cell death in different manner, where damage to the cell membrane is one type of cell death. Cell membrane damage does not exclude that there are other types of cell death in the population of cells.

Principle: Lactate dehydrogenas (LDH) is a stable cytosolic, enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product. The amount of colour formed is proportional to the number of lysed cells

Controls:

- Included in the kit is a positive control for LDH. Dilute 2 µL of the LDH positive control in to 10 mL PBS with 1% BSA. Prepare fresh for each use
- Medium alone without cells.
- Volume correction control (lysis buffer and medium).
- Untreated cells.

3.2.4.4 Reagents

- 96-well plates (Falcon BD Biosciences Discovery Labware 353072)
- Cell culture medium with the appropriate antibiotics but without serum added.
- CytoTox 96® Non-radioactive cytotoxicity assay (Promega G1780)

  *.5 vials of substrate mix

  *.60 mL assay buffer

  * 25 µL LDH positive control

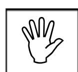
* 3 mL lysis solution (10X)
* 65 mL stop solution
- Multi-channel pipette
- TECAN Infinite® 200 plate reader

3.2.4.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
- Lysis buffer (10X) contains 9% Triton® X-100 (v/v) in water. Triton® X-100 is harmful if swallowed. Causes severe eye irritation. May be harmful if inhaled or in contact with skin. Toxicology not fully investigated. The product may contain traces of ethylene oxide or dioxane, which are probable human carcinogens. Triton® X-100 is dangerous for the environment, avoid therefore putting Triton® X-100 in the sink, use a special waste.
- Wear suitable protective clothing and gloves.

3.2.4.6 Procedure

- Incubate cells on a 96-well plate with different concentrations of nanoparticles for the desired time (24 h is the longest recommended end point).
- Thaw the assay buffer and mix 12 mL assay buffer with one vial of substrate mix. Let the reagents reach room temperature before starting procedure.
- If cells are in suspension or seem not to be adherent, spin the plate for 5min 300g. Transfer 50 µL of the supernatant to an empty 96-well plate (experimental LDH release).
- Wash cells once with serum free media, add 100 µL serum free media to the wells. Add lysis buffer 10 µL/100 µL media and incubate at 37°C for 30-45 min (until cells are completely lysed).
- Transfer 50 µL of the supernatant of the lysed cells to an empty 96-well plate (maximum LDH release).
- Add 50 µL assay buffer to all wells, incubate in dark at room temperature for 30 min.
- Add 50 µL stop solution to all wells. Make sure that there are no bubbles and record the absorbance at 490 using a TECAN Infinite® 200 within 1 h after adding stop solution.
- Calculate: % Cytotoxicity = Experimental LDH / Maximum LDH release



Note: Free nanoparticles could interfere with the readout of the method. Diluted solutions of nanoparticles should be tested in advance.

3.2.4.7 References

(1) http://msds.chem.ox.ac.uk/TR/triton_X-100.html Safety (MSDS) data for Triton X-100 (20090312)

(2) http://www.promega.com/tbs/tb163/tb163.html The company Promega site with technical bulletin (20090312)

(3) Spagnou, S., Miller, A.D. and Keller, M. (2004) Lipidic carriers of siRNA: Differences in the formulation, cellular uptake, and delivery with plasmid DNA. Biochemistry 43, 13348-56.

(4) Hernández, J.M. et al. (2003) Novel kidney cancer immunotherapy based on the granulocytemacrophage colony-stimulating factor and carbonic anhydrase IX fusion gene. Clin. Cancer Res. 9, 1906-16.

3.2.5 SOP NANOMMUNE 3.26 alamarBlue® viability assa

(Partner 10a: Nancy A. Monteiro-Riviere)

3.2.5.1 Purpose

Use of the bioreduction of resazurin to resorufin to measure the viability of cells following timed exposure to nanoparticles.

3.2.5.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the bioreduction of resazurin to resorufin to quantify cell viability following exposure to nanoparticles.

3.2.5.3 Principle

The alamarBlue® cell viability reagent functions as a cell health indicator by using the reducing power of living cells to quantitatively measure the proliferation of cell lines to determine relative cytotoxicity. When cells are alive they maintain a reducing environment within the cytosol of the cell. Resazurin, the active ingredient of alamarBlue® reagent, is a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. The alamarBlue® is placed within the culture wells of 96-well black plates, incubated for 1-4 hours, and fluorescence quantitated (excitation 530-560nm; emission 590nm).

3.2.5.4 Reagents

- alamarBlue® reagent. Invitrogen Cat# DAL1025, 25ml
- Cell culture medium

3.2.5.5 Safety procedures and precautions

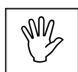
- Standard safety operation procedures are to be followed at all times. Follow safety guidelines provided by the reagent and material manufacturer at all times.
- Prevent contamination of the reagent. Aseptically remove and add to cell culture medium.

3.2.5.6 Procedure

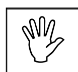
- Plate cells at appropriate concentration in 200µl of medium in the inner wells of a black 96-well plate for 24h. Black wells are necessary to avoid fluorescent interference between

adjacent wells. The outer wells of the plate are filled with media or Hanks' Balanced Salt Solution (HBSS) to help minimize evaporation of the medium on the cells.

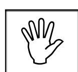
- Expose cells to nanoparticles, negative controls, and positive controls relevant to the treatment protocol at 37°C under cell culture conditions.
- Carefully aspirate treatment medium, pool by treatment, aliquot, and store at -80°C for later cytokine analysis. Add the aB at a 10% concentration to pre-warmed media, add 100µl to each well, and incubate cells for 3h at 37°C under cell culture conditions.
- Remove the plates from the incubator and read fluorescence (top read) in a fluorescent spectrophotometer (excitation wavelength 530-560nm; emission wavelength 590nm).
- Calculate the mean values from multiple wells and normalize to the control (untreated) cells to report as percent cell viability.



- Determine possible interferences of the dye with the nanoparticles.



- Nanoparticle control: Coat wells in 96-well plate with rat tail collagen to help provide adhesion of nanoparticles to the bottom of the wells. Dose wells with nanoparticle concentrations used in viability studies and incubate plates for 24h under cell culture conditions (to mimic viability studies). Centrifuge the plates on the same setting as cells to compact the nanoparticles, carefully aspirate media (leaving nanoparticles in the wells), and add 10% aB medium to the wells for 3h. Centrifuge plate and read fluorescence as above. Any change from control (well with no nanoparticles) indicates nanoparticle interference with the resazurin dye.



- Cell control: Incubate plate containing nanoparticles (as above) in tandem with plate containing cells cultured at appropriate concentration. After 24h, the normal cells are assayed with aB for 3h and the fluorescence quantitated. The plate is centrifuged; the media removed from the wells in the plate containing the nanoparticles, and the aB media is transferred to the wells containing the nanoparticles. The plate is incubated for 1h, centrifuged, the media transferred to wells in a new plate, and the fluorescence quantitated. Any difference between the absorbance from the cells and the absorbance in the nanoparticles indicates nanoparticle interference with the resorufin.

3.2.5.7 Reference

(1) Monteiro-Riviere NA, Inman AO, Zhang LW. Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicology and Applied Pharmacology. 234:222-235, 2009.

(2) Samberg ME, Oldenburg SJ, Monteiro-Riviere NA. Evaluation of silver nanoparticle

toxicity in vivo skin and in vitro keratinocytes. Environmental Health Perspectives 118, 407-413, 2010.

(3) Monteiro-Riviere NA, Oldenburg SJ, Inman AO. Interactions of aluminum nanoparticles with human epidermal keratinocytes. Journal of Applied.Toxicology_30:276-285, 2010.

3.2.6 SOP NANOMMUNE 3.27 CellTiter 96® Aqueous One viability assay
(Partner 10a: Nancy A. Monteiro-Riviere)

3.2.6.1 Purpose

Use of the bioreduction of a tetrazolium compound to measure the viability of cells following timed exposure to nanoparticles.

3.2.6.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the bioreduction of a tetrazolium compound to quantify cell viability following exposure to nanoparticles.

3.2.6.3 Principle

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in cytotoxicity assays. The solution Reagent contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability and is combined with MTS to form a stable solution. The MTS tetrazolium compound (Owen’s reagent) is bioreduced by into a colored formazan product that is soluble in tissue culture medium and presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cellsAssays are performed by adding a small amount of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490nm with a 96-well plate reader.

3.2.6.4 Reagents

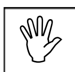
- CellTiter 96® AQueous One Solution Cell Proliferation Assay. Promega Cat# G3589, 20ml
- Cell culture medium

3.2.6.5 Safety procedures and precautions

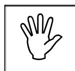
- Standard safety operation procedures are to be followed at all times. Follow safety guidelines provided by the reagent and material manufacturer at all times.
- Prevent contamination of the reagent. Aseptically remove and add to cell culture medium.
- Upon first use, aliquot into sterile 1ml tubes. Return any unused portion to storage at -20°C.

3.2.6.6 Procedure

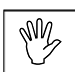
- Plate cells at appropriate concentration in 200µl of medium in the inner wells of a 96-well plate for 24h. The outer wells of the plate are filled with media or Hanks' Balanced Salt Solution (HBSS) to help minimize evaporation of the medium on the cells.
- Expose cells to nanoparticles, negative controls, and positive controls relevant to the treatment protocol at 37°C under cell culture conditions.
- Carefully aspirate treatment medium, pool by treatment, aliquot, and store at -80°C for later cytokine analysis. Add the MTS at a 20% concentration to pre-warmed media, add 100µl to each well, and incubate cells for 3h at 37°C under cell culture conditions.
- Remove the plates from the incubator and transfer MTS to a new 96-well plate to avoid interference with the nanoparticles attached to the cells in the bottom of the wells. Read the absorbance at 490nm.
- Calculate the mean values from multiple wells and normalize to the control (untreated) cells to report as percent cell viability.



- Determine possible interferences of the dye with the nanoparticles.



- Nanoparticle control: Coat wells in 96-well plate with rat tail collagen to help provide adhesion of nanoparticles to the bottom of the wells. Dose wells with nanoparticle concentrations used in viability studies and incubate plates for 24h under cell culture conditions (to mimic viability studies). Centrifuge the plates on the same setting as cells to compact the nanoparticles, carefully aspirate media (leaving nanoparticles in the wells), and add 20% MTS medium to the wells for 3h. Centrifuge plate and read absorbance at 490nm. Any change from control (well with no nanoparticles) indicates nanoparticle interference with the formazan dye.



- Cell control: Incubate plate containing nanoparticles (as above) in tandem with plate containing cells cultured at appropriate concentration. After 24h, the normal cells are assayed with MTS for 3h and the absorbance quantitated. The plate is centrifuged, the media removed from the wells in the plate containing the nanoparticles, and the MTS media is transferred to the wells containing the nanoparticles. The plate is incubated for 1h, centrifuged, the media transferred to wells in a new plate, and the absorbance quantitated. Any difference between the absorbance from the cells and the absorbance in the nanoparticles indicates nanoparticle interference.

3.2.6.7 Reference

(1) Monteiro-Riviere NA, Inman AO, Zhang LW. Limitations and relative utility of screening

assays to assess engineered nanoparticle toxicity in a human cell line. Toxicology and Applied Pharmacology. 234:222-235, 2009.

(2) Samberg ME, Oldenburg SJ, Monteiro-Riviere NA. Evaluation of silver nanoparticle toxicity in vivo skin and in vitro keratinocytes. Environmental Health Perspectives 118, 407-413, 2010.

(3) Monteiro-Riviere NA, Oldenburg SJ, Inman AO. Interactions of aluminum nanoparticles with human epidermal keratinocytes. Journal of Applied.Toxicology_30:276-285, 2010.

3.3 Functionality and Inflammation

3.3.1 SOP NANOMMUNE 3.15v2
Phenotyping of human monocyte derived dendritic cells after exposure to nanoparticles

(Partner 1b: Britta Andersson)

3.3.1.1 Purpose

To determine phenotypical properties of human monocyte derived dendritic cells after exposure to nanoparticles.

3.3.1.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the determination of phenotype of dendritic cells after exposure to nanoparticles.

3.3.1.3 Principle

Dendritic cells express different surface markers during stages of differentiation and maturation. Nanoparticles might influence the maturation of dendritic cells.

3.3.1.4 Reagents and Materials

- 6 well plate (Falcon BD Biosciences Discovery Labware 353046)
- RPMI 1640 medium (Sigma 30255.01), supplemented with 25 µg/mL gentamicin (Gibco Invitrogen Corporation, Paisley, UK), 2 mM L-glutamine (Gibco Invitrogen Corporation), 100 IU/ml penicillin (Gibco Invitrogen Corporation), 100 µg/ml streptomycin (Gibco Invitrogen Corporation), 50 µM β-mercaptoethanol (KEBO-lab, Spånga, Sweden), heat inactivated (56 ºC, 30 min) FCS (HyClone SH30071.03)
- Trypan blue (Sigma-Aldrich T8154) and a Burker chamber
- IL-4 and GM-CSF (PHC0043 and PHC2013, Biosource International)
- Polystyrene FACS tubes (BD Biosciences, 352052)
- Cluster tubes 1.2mL (Corning Incorporated Costar, 4401)
- Phosphate buffered saline pH 7.4
- 96-well plates, round bottom (Falcon BD Biosciences Discovery Labware 353077)
- Disposable plastic pipettes
- Pipette aid

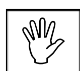
- Conjugated mAbs specific for CD40 (BD Biosciences 555588), CD80 (BD Biosciences 557226), CD83 (BioLegend 305306), CD86 (BD Biosciences 555657), HLA-ABC (BD Biosciences 555552), HLA-DR (BD Biosciences 347400), DC-SIGN (BioLegend 330104) and respective isotype controls.

3.3.1.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class IISafety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.3.1.6 Procedure

- Generate immature MDDC according to SOP 3.05 including the confirmation of phenotype at day 6.
- Count and plate 4×10^5 cells/mL (3mL) in a 6well plate, add nanoparticles at chosen concentrations and LPS as a positive control. Incubate in 37°C with a 6 % CO_2 atmosphere at chosen time points.
- After incubation, transfer the cell culture to FACS tube, centrifuge at 300 g for 5 min.
- In a 96well plate, add 5 uL conjugated mAbs specific for CD40, CD80, CD83, CD86, HLA-ABC, HLA-DR, DC-SIGN and respective isotype controls.
- Discard the supernatant (or save for further experiments), and resuspend the pellet in ice-cold PBS. In the 96well plate, aliquot 100µL per antibody to be analyzed. Incubate for 30 min in the fridge or on ice.
- Wash cells by adding 100µL PBS to each well, centrifuge at 300g for 5min. Resuspend cells in 250µL cold PBS and transfer to small FACS tubes. Analyze the cell surface molecules by flow cytometry.



Note: Prior to exposure of dendritic cells to nanomaterials it is crucial to check the material for endotoxin contamination (e.g. lipopolysaccharide, LPS) (Vallhov et al., Nano Lett. 2006; Oostingh et al., Part Fibre Toxicol 2011). Endotoxins trigger immune responses in dendritic cells that may skew the readout in various assays.

3.3.1.7 References

(1) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, Gabrielsson S. Mesoporous silica particles induce size dependent effects on human dendritic cells. Nano Lett. 2007; 7:3576-82.

(2) Oostingh G, Casals E, Italiani P, Colognato R, Stritzinger R, Ponti J, Pfaller T, Kohl Y, Ooms D, Favilli F, Leppens H, Lucchesi D, Rossi F, Nelissen I, Thielecke H, Puntes VF, Duschl A, Boraschi D. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Particle and Fibre Toxicology 2011; 8(1): 8.

(3) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, Gabrielsson S. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. Nano Lett. 2006; 6(8):1682-6.

3.3.2 SOP NANOMMUNE 3.02v1 Phagocytosis assay with M-CSF activated HMDM and TAMRA-labelled target cells

(Partner 1a: Erika Witasp)

3.3.2.1 Purpose

To study and quantify the phagocytosis of apoptotic target cells by M-CSF-activated primary human macrophages *in vitro*.

3.3.2.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and describes the *in vitro* method of determining the phagocytosis of apoptotic cell engulfment by macrophages by means of fluorescence microscopy

3.3.2.3 Principle

Fluorescently TAMRA-labeled target cells (suspension/non-adherent) are co-cultured with macrophages for 1 h to allow cells to interact. This results in the engulfment of apoptotic target cells by the macrophages. Non-engulfed and/or attached cells are removed from the co-cultures with repeated washing. Phagocytosis is evaluated by counting macrophages in visual light and thereafter counting macrophage-engulfed TAMRA-labeled cells under UV illumination using fluorescence microscope.

3.3.2.4 Reagents and Materials

- 15 ml Falcon tubes (352096)
- 50 ml Falcon tubes (352070)
- Disposable plastic pipettes in 5 ml, 10 ml and 25 ml graduations
- Pipette aid
- Sterile Phosphate buffered saline pH 7.4
- RPMI 1640 medium (Sigma R0833).supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Paisley, United Kingdom)
- RPMI medium (Sigma R0833) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Paisley, United Kingdom)
- TAMRA (5(6)-carboxytetramethyl-rhodamine N-hydroxy-succimide ester, Sigma C4759), 5 mg/ml in DMSO
- 70% denatured alcohol for desinfection

- Micropipette
- Pipette tips (0.5-200 µl, 100-1000 µl)
- Cold Phosphate buffered saline pH 7.4
- Cold 5 mM EDTA
- 4% formaldehyde in PBS
- Hoechst 33342, 1 µg/ml in PBS
- Fluoromount mounting medium (Southern Biotech 0100-01)
- Coverslips, 14 mm in diameter

3.3.2.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all human-derived material as infectious.
- Procedures 'Staining of cells' up until the step of 'Washing and fixation' are to be performed in a Biohazard Class II Safety Cabinet.
- Steps of 'Washing and fixation' and 'Staining and mounting' are to be carried out in a fume hood.
- Lab coats and latex/vinly/nitril gloves must be worn at all times.

3.3.2.6 Procedure

Note: Protect stained cells from direct light during the complete procedure.

3.3.2.6.1 Staining of target cells

- Count cells and pellet $30 \cdot 10^6$ cells in a 50 ml Falcon tube.
- Discard medium and resuspend in pre-warmed RPMI (w/o serum).
- Pellet cells, discard supernatant and repeat one more time.
- Resuspend the cell pellet in 2 ml RPMI (w/o serum).
- Dilute 10 µl TAMRA-stock (5 mg/ml) in 2 ml PBS. (50 µg TAMRA per $30 \cdot 10^6$ cells) in a 15 ml Falcon tube.
- Transfer cell suspension to the tube with TAMRA-solution and mix gently by pipetting up and down a few times.
- Incubate in the dark 15 min at 37ºC. Mix a few times by inverting the tube three times.
- Wash cells three times in RPMI (w/o serum).
- Count cells and resuspend in complete RPMI medium to $2 \cdot 10^6$ cells/ml.
- Plate cells in a 12-well plate, 1 ml per well.

3.3.2.6.2 Treatment of target cells

- Treat cells with pro-apoptotic stimuli. Control cells are incubated in absence of any stimuli for the same time points.

3.3.2.6.3 Washing of target cells

- Spin down TAMRA-labeled target cells after treatment, 1200 rpm for 5 min. Each treatment separately in different 15 ml Falcon tubes.
- Discard supernatant. Resuspend cells in PBS (brought to RT) and pellet cells at 1200 rpm, 5 min.
- Repeat twice.
- Count and adjust cells. Ratio of target cells to macrophages should be approx. 10:1. Assumption: the number of HMDMs in each well is $0.1\text{-}0.3 \cdot 10^6$.

3.3.2.6.4 Preparation of macrophages

- Discard cell culture medium from wells with macrophages. Add RPMI medium (w/o FBS) and discard again to wash out dead floating cells.
- Repeat one more time.

3.3.2.6.5 Co-culture

- Remove medium from macrophages and add target cells in a volume of 0.5 ml complete medium.
- Incubate for 1 hour at 37ºC.

3.3.2.6.6 Washing and fixation

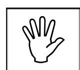
- Wash 8-10 times with cold 5 mM EDTA in PBS, 1 ml per well each time.
- Check in light microscope that all non-attached cells are removed.
- Add 250 µl cold PBS per well.
- Add 250 µl 4% formaldehyde per well (final conc. 2%).
- Fix at RT for 15-20 min or overnight at 4ºC.

3.3.2.6.7 Staining and mounting

- Discard fix-solution and add 1 ml PBS per well.
- Discard PBS and add 200 µl Hoechst staining-solution. Incubate at RT for 15 min.
- Add 1 ml PBS per well and discard again. Mount with coverslips prepared with a drop (5 µl) of Fluormount G mounting medium.
- Allow to settle at RT for 10 min.
- Keep at 4ºC protected from light until analysis.

3.3.2.6.8 Analysis

- In each visual field: first count the number of macrophages in normal light. Start the cell count in the middle of the well and count every cell in a field of view
- Switch to fluorescence light, red filter, and count the number of macrophages with TAMRA-labeled cell/s inside in the same visual field.
- Once all cells are counted move the field of view up the slide vertically and once again count each cell.
- Count at minimum 300-500 macrophages, and at least three visual fields in each well.
- If the edge of the well appears before 300-500 cells are counted move the field of view horizontally and then proceed down vertically counting.
- Calculate the percentage of phagocytosis-positive cells: (total number of phagocytosis positive macrophages divided by the total number of macrophages) multiplied by 100.
- The average number of target cells engulfed by a positive macrophage is an additional data point that can be used.



Note: Before introducing nanomaterials into the assay to investigate potential effects on phagocytosis, it is important to make initial experiments to check for interference of the readout. Suggested control experiments:

1. Expose macrophages alone with the nanomaterial and inspect in light- and fluorescence microscope to check for possible autofluorescence of particles.
2. Expose TAMRA-stained target cells alone with the nanomaterial and analyse in fluorescence microscope to check for possible quenching of the dye.

3.3.2.7 Reference

(1) Kagan VE, Gleiss B, Tyurina YY, Tyurin VA, Elenström-Magnusson C *et al*. (2002) A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. J. Immunol. 169: 487-499

3.3.3 SOP NANOMMUNE 3.11 v1 Detection of caspase-3/7-like activity

(Partner 1a: Erika Witasp)

3.3.3.1 Purpose

To detect caspase3/7-like activity by fluorometric analysis, in apoptotic cells after exposure to nanoparticles.

3.3.3.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection of caspase activity in apoptotic cells after exposure to nanoparticles.

Background/Principle

Background: Apoptosis is a fundamental mode of cell death which performs a regulatory function during normal development, in tissue homeostasis, and in some processes. Apoptosis is primarily mediated by cystein-dependent aspartate-specific proteases called caspases. Caspase-3/7 are so called effector caspases and can be activated by cleavage as the indirect result of receptor ligation and a result of mitochondria activation and apoptosome formation.

Principle: Caspase-3/7 is responsible for the specific proteolytic breakdown of poly (ADP-ribose) polymerase (PARP) by cleavage between Asp 216 and Gly 217 (DEVD 216-217). Based on this Nicholson *et al* (1995) developed a continuous flurorimetric assay with the substrate Ac-DEVD-AMC (Asp-Val-Asp-7-amino-4-methylcoumarin).

Controls:

- Staurosporine is a potential inhibitor of protein kinase C and cells exposed to staurosporine will undergo mitochondria-mediated apoptosis.
- The Fas-activating ab and Fas Ligand induces the trimerization of the Fas-receptors, thereby activating apoptosis.

3.3.3.3 Reagents

- Eppendorf Micro tube 1,5 mM (Sarstedt, 72.690)
- Loose strip black 96-well plate (Corning 3914 Medium binding)
- DEVD-AMC (Acetyl-Asp-Glu-Val-asp-7-Amido-4-*Methyl, A1086, Sigma-Aldrich) dissolved in DMSO
- DTT (threo-1,4-Dimercapto-2,3-butanediol, Cleland’s reagent, D9163, Sigma-Aldrich)

- Nonidet™ P 40 Substitute (74385, Sigma-Aldrich)
- DEVD-AMC substrate Buffer
- 1 x DEVD-AMC substrate Buffer (100mM Hepes , 10% Sucrose, 1% CHAPS):
  2.38g Hepes
  10.0g Sucrose
  100.0mg CHAPS
  Dissolve in 100 ml dH_2O and adjust to pH 7.25-7,3
- 1x PBS (176.8 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4,8.1 mM Na_2HPO_4 x $2H_2O$)
  Staurosporine (Antibiotic AM-2282) (Produkt Nr. S 5921, Sigma). 1 Unit of 0.5 mg, MW: 466.5. Staurosporine is diluted in DMSO (1 mM; 500 µg in 1072 µl DMSO), aliquoted and stored at– 20° C
  Fas-activating antibody (human activating, mouse IgM) (Product Nr. 05-201, clone CH-11, Upstate, Temecula, CA). Formulation: 500µg/ml in PBS, 50% glycerol. The antibody is diluted in media before use: working stock: 10µg/ml; final concentration: 100ng/ml
  TECAN Infinite® 200 plate reader

3.3.3.4 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times
- Wear suitable protective clothing and gloves.

3.3.3.5 Procedure

- Incubate the 0,5 x 106 cells/treatment with different concentrations of nanoparticles for the desired time
- Suggested positive controls: Staurosporine (mitochondria-mediated apoptosis), or Fas antibody (death receptor-mediated apoptosis). Suggested negative control: zVAD-fmk (pan-caspase-inhibitor)
- Collect the cells in 1,5 mL Eppendorf tubes and immediately put on ice
- Wash cells with cold PBS
- Centrifuge and resuspend cells in 50 µl PBS
- Add 25 µl in duplicates of the in PBS re-suspended cells into black 96-well plate on ice
- Store the plate at -20oC for up to one week or analyze immediately
- Start plate reader (for warm-up)

- When analyzing freshly mix DEVD-AMC Buffer. For one complete 96 well plate make 5 mL (5 mL DEVD-AMC substrate Buffer, 25 µL DTT (1 M), 5 µL Nonidet™ P 40 (0,1%), put on ice.
- Add DEVD-AMC salt stock (100 mM in DMSO) to the buffer mix
- Add 50 µL of the DEVD-AMC buffer containing DEVD-AMC, to each well
- Read the plate at excitation waveleght 380 nm and emission wavelength 460 nm using a TECAN Infinite® 200, at 37oC, with a kinetic cycle of 26 timepoints.

Suggested controls for setting up fluorimetrical method:

- DEVD-AMC substrate Buffer including substrate (DEVD-AMC)
- Untreated cells
- Anti-Fas treated cells

3.3.3.6 References

(1) Danial NN and Korsmeyer SJ. 2004. Cell death: critical control points. Cell 116:205-19.

(2) Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Yu VL and Miller K. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37-43

3.3.4 SOP NANOMMUNE 3.24 CaspAce™ assay

(Partner 1b: Britta Andersson)

3.3.4.1 Purpose

To quantify the amount of cells with active caspase (cells undergoing apoptosis) after nanoparticle exposure.

3.3.4.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the quantification of cells with active caspase after exposure to nanoparticles.

3.3.4.3 Principle

Nanoparticles can induce apoptosis, a tightly programmed mechanism of cell death. One characteristic of apoptosis is the activation cascade of caspases (cysteine-aspartic proteases). Pro-caspases are cleaved to its active subunits and are then able to further activate downstream caspases.
The CaspAce™ FITC-VAD-FMK In situ marker is a fluoroisothiocyanate (FITC) conjugate of the cell permeable inhibitor VAD-FMK. It is delivered into the cell where it binds to active caspase.

3.3.4.4 Reagents and Materials

- CaspAce™ FITC-VAD-FMK In situ marker (Promega, G7461 (50 µL) or G7462 (125 µL).
- Phosphate buffered saline (PBS) pH 7.4.
- 96 well u-bottom plate (Falcon BD Biosciences Discovery Labware, 353077)
- Disposable pipettes.
- Multi and single channel pipette aid.
- Cluster tubes 1.2mL (Corning Incorporated Costar, 4401).
- Shaker.

3.3.4.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class IISafety Cabinet.

- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.3.4.6 Procedure

- Expose cells to nanoparticles.
- Harvest cells by centrifugation at 300g for 10 min, the supernatant can be saved for further analysis of for example cytokine release.
- Resuspend cells in PBS and transfer 100 µL cell suspension/well (at least 1×10^5 cells/exposure) into a u-bottom 96-well plate.
- Prepare a 20 µM solution of CaspAce™ FITC-VAD-FMK In situ marker from the stock (5 mM).
- Add 100 µL of 20 µM CaspAce™ FITC-VAD-FMK In situ marker to a final concentration of 10 µM.
- Incubate at 37°C in a humidified atmosphere, in the dark for 20 min. Protect the samples from light for the remaining steps.
- Centrifuge the plate at 300 g, 5 min.
- Discard the supernatant and wash once in 200 µL PBS at 300 g, 5 min.
- Transfer cells to cluster tubes in a final volume of 300 µL PBS.
- Flow cytometry: Acquire at least 1×10^4 cells, in the FL-1 channel.

3.3.5 SOP NANOMMUNE 3.17 v1 Detection of ROS using 2’,7’-dichlorofluorescein (H2DCF)

(Partner 6: Tina Bürki)

3.3.5.1 Purpose

To detect intracellular reactive oxygen species (ROS) induced by nanoparticles.

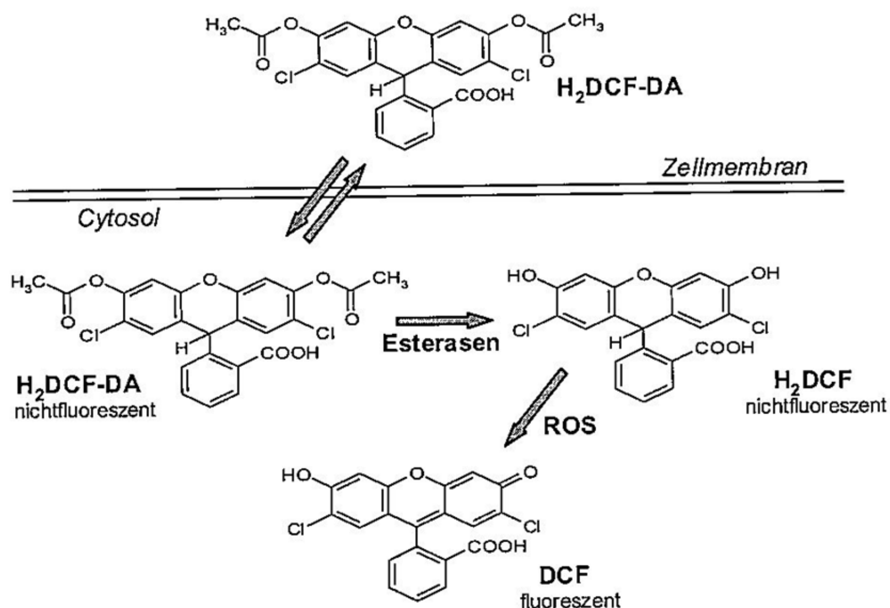
3.3.5.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection of ROS using 2’,7’-dichlorofluorescein (H2DCF).

3.3.5.3 Background/Principle

The recent interest in the role of free radical processes in cellular physiology and pathology has resulted in a demand for methods of quantification of production of ROS in living cells. ROS are very reactive molecules and are therefore extremely unstable, making it impossible to image them directly. Thus detection of ROS relies on detecting end products formed when specific compounds react with ROS. These include measurements of oxidation of dichlorofluorescin (H2DCF), dihydrorhodamine 123 (DHR) or hydroethidine (DHE), H2DCF being used most frequently.

Cell membranes are permeable to non-fluorescent. esterified forms of H2DCF (e.g. H2DCF-DA). This substrate is cleaved in the cytoplasm by intracellular esterases, resulting in non-fluorescent H2DCF that becomes trapped intracellularly. In the presence of ROS, H2DCF is oxidized to green-fluorescent DCF. H2DCF can be oxidized by various ROS including hydrogen peroxide, organic hydroperoxides, nitric oxide and peroxynitrite, thereby serving as an indicator of the degree of general oxidative stress.



Controls:

- SIN-1 (3-morpholinosydnonimine hydrochloride); peroxinitrite-donor

3.3.5.4 Reagents

3.3.5.4.1 *ROS* dye:

- 2',7'-dichlorofluorescin-diacetate; H_2DCF-DA, Molecular Probes.# D-399, MW. 487.29
- Stock solution: 5 mM in DMSO (2.44 mg/ml) ; store at.-20°C
- Final concentration: 50 µM (dilute in HBSS)

3.3.5.4.2 *SIN-1:*

- 3-morpholinosydnonimine hydrochloride.(SIN-1) Sigma# M184, MW. 206.6
- Stock solution: 1 mM in HBSS (0.21 mg/ml); store at.-20°C
- Final concentration: 5 µM (dilute in HBSS)
- Poor stability! Only thaw and dilute before direct application to the cultures

3.3.5.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times

3.3.5.6 Procedure for suspension cells

- transfer required amount of cells to a Falcon tube (200'000/well)
- centrifuge and resuspend pellet in required volume of 50 µM H_2DCF-DA (100 µl/well)
- incubate for 40 min in the incubator at 37°C
- wash pellet 2x with pre-warmed HBSS
- after second wash, resuspend pellet in required volume of HBSS (100 µl/well)

- add 100 µl of the cell suspension per well of a 96 well plate
- add 100 µl of particles (2x concentrated) or SIN-1 (2x concentrated)
- incubate at 37°C in incubator and take measurements after different time points (e.g. 0.5, 1, 2, 3 h) using a fluorescent microplate-reader at 530nm

3.3.5.7 Procedure for adherent cells

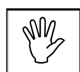
- trypsinize cells and plate 20’000 cells per well of a 96 well plate
- incubate for 24 h in incubator
- remove medium and replace with 100 µl of 50 µM H_2DCF-DA
- incubate for 40 min in the incubator at 37°C
- wash 2x with pre-warmed HBSS
- add 100 µl of the particles or SIN-1 per well of a 96 well plate
- incubate at 37°C in incubator and take measurements after different time points (e.g. 0.5, 1, 2, 3 h) using a fluorescent microplate-reader at 530nm

3.3.5.8 Measuring ROS in the absence of cells

Some nanoparticles with active sites or metal contaminations might produce ROS by a chemical reaction in the absence of cells. To measure the chemically produced ROS, the following protocol can be used to produce H_2DCF from H_2DCF-DA:

- add 1.5 ml 5mM H_2DCF-DA (DMSO stock) to 6 ml MeOH -> 7.5 ml 1mM H_2DCF
- add 30 ml 0.01N NaOH and wrap with a aluminium foil
- mix for 30 min using a magnetic stirrer at room temperature
- stop reaction with 112.5 ml 33mM NaH_2PO_4
- > 150 ml 50 µM H_2DCF
- incubate different concentrations of particles with the esterified dye and measure fluorescence at different time points using a.fluorescent microplate-reader at 530nm

3.3.5.9 Interference of nanoparticles with the test



As this is a fluorimetric test, nanoparticles that reflect or absorb the fluorescent light will affect the DCF results. Possible controls include:

- One sample can be included where nanoparticles at the highest concentration used in the assay are present but no cells. No signal should be measured in this well to exclude interference of nanoparticles just by their ability to reflect/absorb fluorescent light.

- A test can be performed if the nanoparticles create ROS by themselves and not via their effect on the redox-systems in the cells. For this, a specific protocol is required that converts the H2DCF-DA to H2-DCF which can react with ROS (see e.g. Limbach et al. 2007)

3.3.5.10 References

(1) Cathcart R, Schwiers E, Ames BN. 1983. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Anal Biochem 134(1):111-6.
(2) Voelkel K, Krug HF, Diabate S. 2003. Formation of reactive oxygen species in rat epithelial cells upon stimulation with fly ash. J Biosci 28(1):51-5.
(3) Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, Stark WJ. (2007). Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ Sci Technol 41,4158-63.

3.3.6 SOP NANOMMUNE 3.19 v2
Detection of cytosolic superoxide using dihydroethdium (DHE)

(Partner 1a, 1b and 6: Andrea Kunzmann, Britta Andersson, Tina Bürki)

3.3.6.1 Purpose

To detect cytosolic superoxide production induced by nanoparticles

3.3.6.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection of cytosolic superoxide using dihydroethdium (DHE; also called hydroethidine).

3.3.6.3 Background/principle

The redox-sensitive, cell-permeable fluorophore DHE is used to evaluate the cytosolic production of superoxide. DHE is oxidized by superoxide to 2-hydroxyethidium which binds to DNA leading to enhanced intracellular fluorescence (excitation 500-530 nm, emission 590-620 nm)

Possible positive control: DMNQ (2,3 – Dimethoxy- 1,4 – naphthoquinone); Redox-cycling agent that induces intracellular superoxide anion formation.

3.3.6.4 Reagents

- DHE: Molecular probes, D23107 (5mM stock in DMSO). Final concentration: 5µM in cell culture media
- DMNQ (Alexis Biochemicals, ALX-420-027-M005).). Final concentration: 100µM in cell culture media

3.3.6.5 Safety procedures and precautions

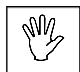
- Standard safety operation procedures are to be followed at all times

3.3.6.6 Procedure

- Plate 1 x 106 cells (primary cells) or 1.5 x 105 cells (cell lines) in a 24 well plate (final volume 1ml)
- Expose the cells to different concentrations/time points to nanoparticles
- Wash cells 2 x with PBS
- Incubate cells with 5µM DHE in medium for 45 min at 37°C (final volume 0.5ml)
- Wash cells 2 x with PBS

- Harvest cells, put them on ice
- Keep cells on ice until FACS analysis (FL-3)

3.3.6.7 Control for assay-nanoparticle interactions



To assure that addition of nanoparticles will not interfere with the read out of the assay control experiments should be carried out.

- Examine if the particles show autofluorescence and if they can be detected at the same wavelength as the dye.
- To investigate possible quenching of the dye, examine the absorbance spectrum of the nanoparticles and check that the absorbance peak does not overlap with either the excitation or emission peak of the oxidized dye (Oostingh et al., Part Fibre Toxicol 2011).
- Incubate nanoparticles in cell-free medium at different concentrations with the parent probe or the oxidized metabolite respectively. Compare the fluorescence intensity to a control solution of the dye and measure fluorescence at different time points using a fluorescent microplate-reader at excitation/emission wavelengths of 500-530 nm/590-620 nm, respectively.

3.3.6.8 References

(1) Oostingh G, Casals E, Italiani P, Colognato R, Stritzinger R, Ponti J, Pfaller T, Kohl Y, Ooms D, Favilli F, Leppens H, Lucchesi D, Rossi F, Nelissen I, Thielecke H, Puntes VF, Duschl A, Boraschi D. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Particle and Fibre Toxicology 2011; 8(1): 8.

(2) Zhao, H., S. Kalivendi, Zhang H, Joseph J, Nithipatikom K, Vásquez-Vivar J, Kalyanaraman B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic Biol Med 2003; 34(11): 1359-68.

(3) Zielonka J, Vasquez-Vivar J, Kalyanaraman B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. Nat Protoc. 2008;3(1):8-21

3.3.7 SOP NANOMMUNE 3.20 v1 Detection of mitochondrial superoxide using Mitosox

(Partner 1a: Andrea Kunzmann)

3.3.7.1 Purpose

To detect superoxide production induced by nanoparticles

3.3.7.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection of mitochondrial superoxide using dihydroethdium (DHE).

3.3.7.3 Background/Principle

Mitochondrial superoxide is generated as a by-product of oxidative phosphorylation. In an otherwise tightly coupled electron transport chain, approximately 1–3% of mitochondrial oxygen consumed is incompletely reduced; these "leaky" electrons can quickly interact with molecular oxygen to form superoxide anion, the predominant reactive oxygen species in mitochondria. MitoSOX Red mitochondrial superoxide indicator is a cationic derivative of dihydroethidum designed for highly selective detection of superoxide in the mitochondria of live cells. The cationic triphenylphosphonium substituent of MitoSOX Red indicator is responsible for the electrophoretically driven uptake of the probe in actively respiring mitochondria. Oxidation of MitoSOX Red indicator by superoxide results in hydroxylation at the 2-position (**Figure 1**). 2-hydroxyethidium (and the corresponding derivative of MitoSOX Red indicator) exhibit a fluorescence excitation peak at ~400 nm that is absent in the excitation spectrum of the ethidium oxidation product generated by reactive oxygen species other than superoxide.

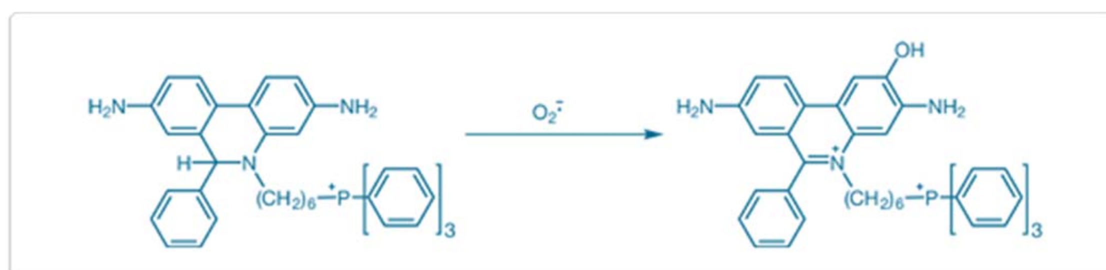


Figure 1. Oxidation of MitoSox Red mitochondrial superoxide indicator to 2-hydroxy-5-(triphenylphosphonium)hexylethidium by superoxide ($\bullet O_2^-$).

3.3.7.4 Reagents

Mitosox (Invitrogen M36008; 10x 15µg/ml)

– Stock solution: 1 vial Mitosox + 13µl DMSO (=5mM stock solution)

- positive control: cumene hydroperoxide (CuOOH) (Sigma-Aldrich 247502, 80%)
- Stock: 80%, MW=152.2 (5µl in 2.7ml media = 10mM; 5µl in 1ml = 50µM)

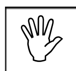
3.3.7.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times

3.3.7.6 Procedure

- Plate 1 x 10^6 cells (primary cells/cell line) in a 24 well plate (final volume 1ml)
- Expose the cells to different concentrations/time points to nanoparticles'
- Positive control: 50µM cumene hydroperoxide (CuOOH), 1h, 37°C
- Wash cells 2 x with PBS
- Incubate cells with 5µM Mitosox in media for 10-15 min at 37°C (final volume 1 ml)
- Wash cells 2 x with PBS
- Harvest cells, put them on ice
- Keep cells on ice until FACS analysis (FL-2)

3.3.7.7 Control for assay-nanoparticle interactions



To assure that addition of nanoparticles will not interfere with the read out of the assay control experiments should be carried out.

- Examine if the particles show autofluorescence and if they can be detected at the same wavelength as the dye.
- To investigate possible quenching of the dye, examine the absorbance spectrum of the nanoparticles and check that the absorbance peak does not overlap with either the excitation or emission peak of the oxidized dye (Oostingh et al., Part Fibre Toxicol 2011).
- Incubate nanoparticles in cell-free medium at different concentrations with the parent probe or the oxidized metabolite respectively. Compare the fluorescence intensity to a control solution of the dye and measure fluorescence at different time points using a fluorescent microplate-reader at excitation/emission wavelengths of 500-530 nm/590-620 nm, respectively.

3.3.7.8 References:

(1) Batandier, C., E. Fontaine, et al. Determination of mitochondrial reactive oxygen species: methodological aspects. J Cell Mol Med 2002;6(2): 175-87.
(2) Kudin, A. P., N. Y. Bimpong-Buta, et al. Characterization of superoxide-producing sites in isolated brain mitochondria. J Biol Chem 2004;279(6): 4127-35.

(3) Oostingh G, Casals E, Italiani P, Colognato R, Stritzinger R, Ponti J, Pfaller T, Kohl Y, Ooms D, Favilli F, Leppens H, Lucchesi D, Rossi F, Nelissen I, Thielecke H, Puntes VF, Duschl A, Boraschi D. Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Particle and Fibre Toxicology 2011; 8(1): 8.

3.3.8 SOP NANOMMUNE 3.13 v2
Lipid oxidation analysis by FACS with BODIPY dye
(Partner 1: Jingwen Shi)

3.3.8.1 Purpose

To study and quantify lipid oxidation in cells

3.3.8.2 Scope

This protocol is available for all members of the NANOMMUNE project and describes the method of determining the level lipid oxidation by the fluorescent probe 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, or di(acetoxymethyl ester) (BODIPY).

3.3.8.3 Background and principle

The redox sensitive dye BODIPY (a fatty acid analog) integrates into biomembranes of cells. In the event of oxidative stress BODIPY becomes oxidized. Once oxidized, both the absorption and emission maxima of the BODIPY$^{581/591}$ (Invitrogen, C2938) probe are blue-shifted with good spectral separation of the non-oxidized (red fluorescent, FACS FL-2) and oxidized (green fluorescent, FACS FL-1) forms. The shift in fluorescence can be measured with a flow cytometer, fluorometer, or fluorescence microscope. Equivalent substrates based on the same principle for other oxidation processes (are also available (e.g. from Invitrogen).
Note: BODIPY is an indirect indicator of lipid peroxidation, and may cause artifacts. For example, the stimulus may directly oxidize the BODIPY probe (even catalyzed by various enzymes in cells) in the absence of ROS production inside cells. Therefore, it is important to verify ROS production inside cells with assays such as DCF.

3.3.8.4 Reagents and Materials

- BODIPY (di(acetoxymethyl ester), Invitrogen, C2938).
- Dimethylform-amide, DMF.
- Phosphate buffered saline, PBS.
- Flow cytometer, FACS.

3.3.8.5 Procedure

Working solutions should be freshly prepared. Protect BODIPY from direct light and keep on ice to avoid further artificial oxidation. The BODIPY concentration should be kept as low as possible to avoid potential artefacts from overloading such as incomplete hydrolysis.

The following protocols provide general guidelines derived from manufacturer's guidelines, and should be modified for the particular application and sensitivity required.

- plate 1 $*10^5$ cells per ml per well in 12-well plates
- Shortly before performing the experiments, reconstitute the ROS indicator to make a concentrated stock solution. Keep tightly sealed until ready to use.
- Remove cells from growth media via centrifugation or pipetting. Resuspend cells in prewarmed PBS containing the probe to provide a final working concentration of ~1–10 mM dye. **The optimal working concentration and labeling time for your application must be empirically determined.**
- Incubate cells with 5µM BODIPY in tissue culture medium at 37ºC 30 min. prior to harvesting. (Incubate at the optimal temperature for the cells. Generally, a loading time of 5–60 minutes is sufficient.)
- Remove the loading buffer; return the cells to prewarmed growth medium.
- Spin down at 1200rpm 5min, discard the supernatant.
- Wash the pellet in PBS.
- Resuspend the cells in 0,5 ml cold PBS, put tubes on ice.
- Analyze on FACS in FL-1 and FL-2 detector: Determine the baseline fluorescence intensity of a sample of the loaded cells prior to exposing the cells to experimental inducements. Any extracellularly bound dye can be quenched using Trypan Blue (~0.0025% w/v) in order to better distinguish the signal from the intracellular ROS response.

3.3.8.5.1 Negative controls should be assessed as follows:

- Examine unstained cells for autofluorescence in the green emission range.
- For flow cytometry, ascertain that the forward and side scatter of cells is unchanged after dye-loading and treatment. Changes in cell dimensions may be related to blebbing or shrinkage resulting from handling or a toxic response.
- Examine the fluorescence of cell-free mixtures of dye and buffer/media with and without the inducer/stimulant. In the absence of extracellular esterases and other oxidative enzymes, the ROS indicator should exhibit a gradual increase in fluorescence over time, which may be related to spontaneous hydrolysis, atmospheric oxidation, and/or light-induced oxidation.
- Examine the fluorescence of untreated loaded cells that have been maintained in growth medium or simple buffer. In healthy cells, oxygen radicals are eliminated by cellular enzymes and/or natural antioxidants. Following the dye-loading recovery period, healthy cells should exhibit a low level of fluorescence that is relatively stable for the duration of the

experiment; however, a gradual increase (due to auto-oxidation) or decrease (due to loss of dye from cells or photobleaching) in fluorescence may be observed. In the absence of any stimulus or inducement, a burst of fluorescence in healthy, untreated cells could indicate progress to cell death or some other oxidative event.

3.3.8.5.2 To create positive controls, oxidative activity may be stimulated with:

- PMA; stock solution 1 mM in DMF; working concentration 100 pM to 10 mM)
- the bacterial chemotatic peptide N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP; stock solution 1 mM in DMF; working concentration 1–10 mM)
- H_2O_2 or tert-butyl hydroperoxide (TBHP) to a final concentration of ~100 mM (increase or decrease based on the sensitivity and response of the cells).

To assure that an added drug or compound will not quench the dye, examine the absorbance spectrum of the compound and determine that the absorbance peak does not overlap with either the excitation or emission peak of the oxidized dye. Alternatively, you can mix a solution of the drug/compound with carboxy-DCF (C368), fluorescein (F1300), or calcein (C481), then compare the fluorescence intensity to a control solution of the dye, or subject a culture loaded with the cell-permeant, oxidized form of the dye to the drug/compound and compare to dye-loaded cells untreated.

3.3.8.6 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
- Wear suitable protective clothing and gloves.
- Work preferably in a fume hood.
- Extra caution required when handling DMF which is harmful by inhalation, ingestion or skin contact. It may act as a carcinogen. Ingestion or absorption through skin may be fatal. Exposure may result in foetal death. Long-term exposure may result in kidney or liver damage. In addition it is an irritant.
- When handling BODIPY extra caution needs to be taken. Use appropriate protective equipment and methods to clean up spilled substances promptly. Absorb spill onto an appropriate material. Collect and dispose of all waste in accordance with applicable laws.
- Do not allow BODIPY to reach ground water, water course, or sewage system.

3.3.8.7 References

(1) Seiler et al, Cell metabolism, 2008, 8, 237-248

(2) Invitrogen, MSDS Reactive Oxygen Species (ROS) Detection Reagents

3.3.9 SOP NANOMMUNE 3.21 v1 Mitochondrial membrane potential (MMP) measured by TMRE

(Partner 6: Tina Bürki)

3.3.9.1 *Purpose*

To test mitochondrial membrane potential (MMP) after exposure to nanoparticles.

3.3.9.2 *Scope*

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection mitochondrial membrane potential (MMP) after exposure to nanoparticles.

3.3.9.3 *Principle*

An early indication of apoptosis involves a collapse in the electrochemical gradient across the mitochondrial membrane. Moreover, mitochondria are very susceptible to apoptosis induced by oxidative stress. Loss of mitochondrial membrane potential can be detected by a unique fluorescent cationic dye known as TMRE (tetramethylrhodamine ethyl ester). TMRE easily penetrates cells and enters the mitochondria. It aggregates in the mitochondria of non-apoptotic cells and fluoresces bright orange/red, whilst in apoptotic cells it diffuses throughout the cell. Once dispersed, the reagent assumes a monomeric form and exhibits a reduced orange/red fluorescence level. This allows an easy distinction between apoptotic and non-apoptotic fluorescent cells which can be read with a flow cytometer,

3.3.9.4 *Reagents*

- TMRE (Fluka, 87917); stock solution 25µM in DMSO/Ethanol
- 1x PBS (176.8 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4,8.1 mM Na_2HPO_4 x $2H_2O$)
- Fas-activating antibody (human activating, mouse IgM) (Product Nr. 05-201, clone CH-11, Upstate, Temecula, CA). Formulation: 500µg/ml in PBS, 50% glycerol. The antibody is diluted in media before use: working stock: 10µg/ml; final concentration: 100ng/ml

3.3.9.5 *Safety procedures and precautions*

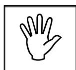
- Standard safety operation procedures are to be followed at all times.
- Follow safety guidelines provided by the reagent and material manufacturer at all times.

3.3.9.6 Procedure

- Incubate cells (up to 1 x 10^6/ml) with different concentrations of nanoparticles for the desired time (e.g. 1.5 x 10^5 Jurkat cells in 24 well plates in 1ml)

Suggested controls: Induce apoptosis with Fas antibody (Jurkat cells: 100ng/ml for 5 h; this concentration and time might have to be optimized for other cell types)

- Collect the cells in a centrifugation tube
- Wash cells with cold PBS
- Resuspend pellet with 500 µl 25 nM TMRE
- Incubate for 10 min at 37°C
- Centrifuge for 3 min at 4000 rpm
- Wash pellet with 1x PBS
- Resuspend pellet with 300 µl PBS
- Analyze by FACS sorting in FL2



Note: Before introducing nanomaterials into the assay to investigate potential effects on the mitochondrial membrane potential, it is important to include controls to check for potential interference with this assay.
Suggested control experiments:

- Include one sample adding the highest concentration of nanomaterials used for the assay but without cells. This sample is treated equally to all the other experimental samples, and FACS measurement is performed for approximately the same time as it takes for 10’000 cells. This control will give a feeling if nanoparticle aggregates/agglomerates appear in the scatter plot and if these are stained with the TMRE dye.

3.3.10 SOP NANOMMUNE 3.09 v1
Fpg-comet assay to analyze DNA damage
(Partner 1a: Jingwen Shi)

3.3.10.1 Purpose

To study and quantify DNA oxidative damage in response to genotoxic exposure in *ex vivo* model systems.

3.3.10.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and describes the *ex vivo* method of determining the level of oxidative DNA damage in eukaryote cells by means of microgel electrophoresis and fluorescence microscopy

3.3.10.3 Background and principle

Generation of DNA damage is considered to be an important event in carcinogenesis. The simple version of the comet assay detects DNA migration caused by strand breaks, alkaline labile sites, and transient repair sites. By incubation with bacterial glycosylase/endonuclease enzymes such as 8-oxodguanine (8oxodG), formamidopyrimidine DNA glycosylase (fpg) and endonuclease III (endoIII), broad classes of oxidative DNA damage, alkylations, and ultraviolet light-induced photoproducts are detected as additional DNA migration.

3.3.10.4 Principle

In the simple comet assay, single cell suspensions are embedded in agarose and lysed. Following alkaline treatment (depending on the pH of the alkaline treatment, some DNA lesions called alkaline labile sites are converted to strand breaks) and electrophoresis, DNA migrates toward the anode in a manner that is dependent on the number of lesions in the nucleoids. The extent of migration is visualized in a fluorescence microscope after staining of the DNA.

Detection of particular sites is carried out by digestion of the nucleoids with bacterial DNA repair enzymes. One of the most important lesions detected by the fpg protein is the 7,8-dihydro-8-oxo-deoxyguanosine (8oxodG) lesion, which is a major product of oxidative stress with clear mutagenic potential.

3.3.10.5 Strenghts and limitations

The simple comet assay, developed by N.P. Singh, combines the simplicity of biochemical techniques with the single cell approach typical of cytogenetic assays. The method is sensitive and virtually any eukaryote cell population is amenable to analysis. However, it is important that

the performance of the assay is investigated in multi-laboratory validation trials. Also, the predictive value of the assay is unknown because it has not been investigated in prospective cohort studies.

There are several enzyme-linked modified comet assays: the fpg-comet assay mainly measures oxidized purines (mainly 8oxodG), and the endoIII-comet assay mainly measures oxidized pyrimidines. In view of its importance and relative ease to measure, 8oxodG has come to be commonly regarded as a good and sensitive marker of oxidative damage to DNA. A potential problem with the use of enzymes is that they may not detect all the potential substrate, and therefore reaction conditions need to be optimized.

The readout of oxidized DNA product by damage-specific antibodies, HPLC and mass spectrometry based techniques have many advantages, however estimates may be artificially high due to oxidation during isolation, storage or hydrolysis of DNA.

3.3.10.6 Reagents and Materials

- Microscope object slides, 14 mm in diameter (Histolab 06275)
- Coverslips, 14 mm in diameter (Fischer 12-545F)
- Bovine serum albumin (BSA) (A7906)
- Disodium EDTA (Sigma E5134)
- Ethidium Bromide (E8751)
- Formamidopyrimidine DNA glycosylase (Fpg-enzyme) (from Prof. Andrew Collins)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (H3375)
- Low Melting Point Agarose (LMPA) (Fischer BP165-25)
- Normal Melting Agarose (NMA) (Fischer BP1365-100)
- Phosphate Buffered Saline (PBS) (Ca^{2+}, Mg^{2+} free) (GIBCO 450-1300EC)
- Potassium Chloride (KCl) (P5405)
- Sodium Chloride (NaCl) (Sigma S9625)
- Sodium Hydroxide (NaOH) (Sigma S5881)
- Triton X-100 (Sigma x-100)
- Trizma Base (Sigma T8524)
- Agarose gels

Prepare 0.3% NMA and 0.75% LMPA in PBS. Microwave until the agarose dissolves. For NMA, precoat slides with 30µl per field at least one day in advance, dry at room temperature, avoid high humidity conditions.

For LMPA, aliquot 1-5ml samples per tube and refrigerate until needed. When needed, briefly melt agarose in microwave oven and keep in 37°C water bath to cool and stabilize the temperature.

3.3.10.6.1 Lysis solution (store at room temperature)

- 2.5M NaCl
- 10mM Tris
- 0.1M EDTA
- Set pH to 10 with 10M NaOH
- Add 1% Triton X-100 just before use

3.3.10.6.2 Fpg enzyme reaction buffer (store at 4ºC)

- 0.1M KCl
- 0.5 mM EDTA
- 40 mM HEPES
- 0.2 mg/ml BSA
- Adjust to pH 7.8-8.0 with KOH
- Filter the solution (CN 0.45)

3.3.10.6.3 Fpg enzyme (store at -80ºC)

- Electrophoresis solution (store at room temperature)
- 0.3 M NaOH
- 1 mM EDTA

3.3.10.6.4 Neutralization buffer (store at room temperature)

- 0.4M Tris
- Adjust to pH 7.5 with concentrated HCl

3.3.10.6.5 Staining solution (store at room temperature)

- Ethidium bromide (0.2µg/ml, diluted in dH_2O)

3.3.10.7 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Lab coats and latex/vinly/nitril gloves must be worn at all times
- Wear goggles when making electrophoresis buffer for alkaline treatment.
- Ethidium bromide is toxic, handle with care, wear gloves and throw pipette tips in special container.

3.3.10.8 Procedure

Note: Protect from direct light and keep on ice to avoid further artificial oxidation.

3.3.10.8.1 Standard curve for determination of fpg enzyme concentration

Use H_2O_2 as positive control, and treat cells with a range of concentrations up to 100µM, incubate 30min. Perform the comet assay (see standard protocol as below, section 7.2) and add enzyme at different dilutions (e.g. 1 in 300, 1000, 3000, 10000, 30000). At the desired enzyme concentrations, the enzyme reveals oxidized bases as breaks in a H_2O_2-dose-dependent manner, across a ‘plateau’ of enzyme concentrations.

3.3.10.8.2 Standard protocol for fpg-comet assay

3.3.10.8.2.1Preparation

- Precoat slides with 0.3% NMA, at least one day in advance.
- Prepare all solutions.
- Melt 0.75% LMPA and keep at 37°C water bath.
- Get some ice.

3.3.10.8.2.2Prepare cells

- For monolayer cultures, plate ~5×10^5 cells/well in 12-well plates. For suspension cultures, plate accordingly.
- Note: Cell number can be changed for optimal visualization density under microscope
- Expose to desired agent.
- When harvesting, remove cell medium and wash 2 x 1 ml PBS.
- Add 80 µl trypsin:EDTA and remove ~60 µl. Incubate 5 min.
- Add 200 µl cell medium and pipette into 1.5ml Epp-tubes on ice. (Control cytotoxicity with trypan blue; dead cells give false positive results)
- Centrifuge at 1400rpm for 3 min and remove supernatant.
- Wash with 1 ml PBS, centrifuge again and remove supernatant.
- Resuspend in 200 µl PBS.

3.3.10.8.2.3Procedure

- Prepare gels**:**

  Add 27 µl cell suspension to 210 µl 37 °C LMPA.

  Spread 30 µl of the gel over the field for reaction with and without enzyme, respectively, using the pipette. Wait until it dries before next step.

  Put the slides in lysis buffer for 1 h or overnight on ice in dark.

- Fpg enzyme buffer:
  Put the slides in fpg enzyme buffer for 3 x 5 min, on ice in dark.
  Wipe off the slides and put on ice.
  Thaw Fpg-enzyme and dilute to appropriate concentration.
  Add 30 μl enzyme buffer or 30 μl diluted fpg-enzyme to each field of the slides, respectively.
  Incubate in a humidity chamber, 37°C for 30 min.
- Alkaline treatment:
  Put the slides in electrophresis buffer for 40 min, on ice in dark.
- Electrophoresis:
  Perform electrophoresis (~25 V) in the same solution (cold), 30 min in dark.
- Neutralization:
  Wash the slides in neutralization buffer for 2 x 5 min and in water for 5 min, dry over night, room temperature.
- Fixation:
  Fix in methanol for 5 min and dry, room temperature.
  Add 10μl ethidium bromide per field. Put coverslips and analyze.

3.3.10.9 Analysis

- For visualization of DNA damage, observations are made of stained-DNA using a 40× objective on a fluorescent microscope.
- Score manually total number of cells positive for comets. Can also score respective number of cells positive for different categories of comets according to the Kabayashi classification. Generally, 50 to 100 randomly selected cells are analyzed per sample.

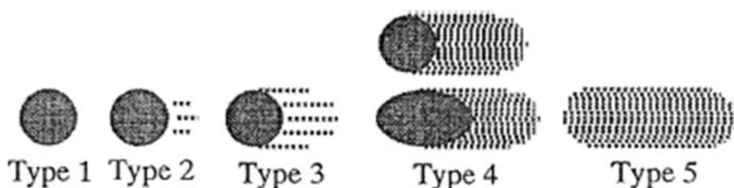
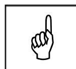


Fig. 3 Schematic of visual classification of comets by Kobayashi et al. (1995). They represent comets of Types 1 to 5

- Software can also be used to quantitate the length of DNA migration (diameter of comet) and the percentage of migrated DNA (percent DNA in the tail, %T). It is also popular to express in terms of tail moment, which is the tail length multiplied with %T. Generally, 50 to 100 randomly selected cells are analysed per sample.

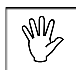
- Fpg-comets are defined as the additional DNA damage from fpg-enzyme treated sample to no enzyme treated sample.



Note: With the fpg-comet assay, both DNA damage in terms of DNA fragmentation and oxidative DNA damage can be evaluated.

- Statistical analysis for in vitro data is based on multiple cultures.

3.3.10.10 Control for assay-nanoparticle interactions



To exclude possible interactions of nanoparticles with the comet assay a number of considerations need to be taken into account. The main concern for interaction is that particles can be seen in the “head” of the comets in the microscope; for a comprehensive review please refer to Karlsson H.L. 2010.

3.3.10.11 Reference

Møller P. The alkaline comet assay. Towards validation in biomonitoring of DNA damaging exposures. Basic & Clinical Pharmacology & Toxicology 2006; 98:336-345.

Collins A.R., Dusinska M., Gedik C.M., Stetina R. Oxidative damage to DNA: do we have a reliable biomarker? Environmental Health Perspectives 1996; 104:465-469.

Karlsson H.L. The comet assay in nanotoxicology research. Analytical and Bioanalytical Chemistry 2010; 398:651-666.

3.3.11 SOP NANOMMUNE 3.23 TUNEL assay

(Partner 1b: Britta Andersson)

3.3.11.1 *Purpose*

To quantify the amount of cells with fragmented DNA (apoptotic cells) after nanoparticle exposure.

3.3.11.2 *Scope*

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the quantification of cells with fragmented DNA after exposure to nanoparticles.

3.3.11.3 *Principle*

Nanoparticles can induce apoptosis, a tightly programmed mechanism of cell death. One of the hallmarks of apoptosis is DNA fragmentation. Cleavage of genomic DNA during apoptosis may yield double stranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks ("nicks") in high molecular weight DNA. Those DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. The TUNEL method consist of 2 steps. 1) Labeling of DNA strand breaks by Terminal deoxynucleotidyl transferase (TdT) which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner. 2) TMR red labeled nucleotides, incorporated in nucleotide polymers, are detected and quantified by flow cytometry.

3.3.11.4 *Reagents and Materials*

- In situ cell death detection kit, TMR red Cat no. 12 156 792 910 Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany.
- Phosphate buffered saline (PBS) pH 7.4.
- Paraformaldehyde 4 % in PBS.
- Ice cold methanol minimum 70 %.
- 96 well u-bottom plate (Falcon BD Biosciences Discovery Labware 353077)
- Disposable pipettes.
- Multi and single channel pipette aid.
- Cluster tubes 1.2mL (Corning Incorporated Costar, 4401).
- Shaker.

3.3.11.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class IISafety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.3.11.6 Procedure

- Expose cells to nanoparticles, the TUNEL assay requires at least 0.5×10^6 cells/condition since cells are easily lost during washing procedures.
- Harvest cells by centrifugation at 300g for 10 min, the supernatant can be saved for further analysis of for example cytokine release.
- Resuspend cells in PBS and transfer 100 µL cell suspension/well into a u-bottom 96-well plate.
- Fixation of cells: Add 100 µL/well of 4 % paraformaldehyde (to a final concentration of 2 %). Resuspend well and incubate at room temperature for 60 min on a shaker to avoid clumping of cells.
- Permeabilizing of cells: Centrifuge 5 min at 300 g and discard the paraformaldehyde. Add 100 µL ice-cold methanol and incubate overnight in -20°C.
- Centrifuge the plate at 300g for 10 min and remove the supernatant by using vacuum suction.
- Wash twice with PBS (200 µL/well). If there is a substantial loss of cells during the washing steps, this can be reduced to one washing step.
- Prepare the TUNEL mix, using vial 1 and 2. The manufacturer's instructions uses 50 µL/sample, however this can be titrated to save components. The TUNEL mix should be prepared freshly and stored on ice until use.
- Remove 100 µL of Label solution (vial 2) for two negative controls.
- Add 50 µL of Enzyme solution (vial 1) to the remaining 450 µL Label solution in vial 2 to obtain 500 µL TUNEL reaction mixture, enough for 10 samples.
- Negative control: incubate fixed and permeabilized cells in Label solution 50 µL/well
- Incubate fixed and permeabilized cells with 50 µL TUNEL mix and incubate for 60 min at 37°C in a humidified atmosphere, in the dark.
- Wash samples twice with PBS 200 µL/well.

- Transfer cells to cluster tubes in a final volume of 300 μL PBS.
- Flow cytometry: Acquire at least 1×10^4 cells, in the FL-2 channel

3.3.12 SOP NANOMMUNE 3.16v3 Cytokine measurement by ELISA

(Partner 1a, 1b and 6: Andrea Kunzmann, Britta Andersson, Tina Bürki)

3.3.12.1 Purpose

To determine cytokine release from primary human monocyte derived dendritic cells or macrophages or the Jurkat T cell line after exposure to nanoparticles.

3.3.12.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the determination of cytokine from primary human monocyte derived dendritic cells or macrophages or the Jurkat T cell line after exposure to nanoparticles.

3.3.12.3 Principle

Dendritic cells, macrophages and T lymphocytes release cytokines in order to communicate with other cells within the immune system. Nanoparticles might influence the release of cytokines.

Enzyme-linked Immunosorbent Assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: The ELISA can be used to detect the presence of proteins that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen. An ELISA is a five-step procedure: 1) coat the microtiter plate wells with antigen; 2) block all unbound sites to prevent false positive results; 3) add antibody to the wells; 4) add anti-mouse IgG conjugated to an enzyme; 5) reaction of a substrate with the enzyme to produce a colored product, thus indicating a positive reaction.

3.3.12.4 Reagents and Materials

3.3.12.4.1 Primary dendritic cells or macrophages

- ELISA kit for *cytokine of interest* (IL-10, IL-12, TNF-α) (MABTECH AB)
- Phosphate buffered saline (PBS) pH 7.4
- PBS with 0.05% Tween 20 (PBS-Tween)
- PBS-Tween with 0.1% BSA (Incubation buffer)
- Nunc-Immuno Plates, MaxiSorp (439454, Nunc, Thermo Fisher Scientific)

- Substrate buffer (500mL, store dark in fridge for up to 6 months)
  1. Dissolve 50.5 mg $MgCl_2$ x $6H_2O$ in 400 mL MQ H_2O.
  2. During continuously stirring add 48.5 mL $C_4H_{11}NO_2$ (diethanolamin).
  3. Add 0.5 mL NaN_3 (20% solution).
  4. Adjust pH to 9.8 by adding HCl.
  5. Add MQ H_2O up to 500mL.
- Phosphatase Substrate 5 mg tablets (S0942, Sigma, pNPP)
- Disposable plastic pipettes
- Pipette aid

3.3.12.4.2 *Jurkat T* cells

- ELISA kit for cytokine of interest (IL-2) (eBiosciences; 88-7026-86)
- Phosphate buffered saline (PBS) pH 7.4

3.3.12.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class II Safety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.3.12.6 Procedure

3.3.12.6.1 Primary dendritic cells or macrophages

- Expose cells to nanoparticles. For MDDC: seed $0.4x10^6$ cells/ml. For HMDM: seed $1x10^6$ cells/ml in 96 well plates (200µl culture volume) or 24 well plates (1ml culture volume).
- For MDDC: Collect floating and adherent cells after cooling the culture plate on ice for 10-15 min. Spin the cells for 5 min at 300g and harvest the supernatant.For HMDM: Harvest supernatants from cells exposed to nanoparticles by spinning down the cell culture plate for 5 minutes at 1500 rpm.
- Store supernatants in case needed at -70°C, preferably in small aliquots.
- Coat an ELISA plate with mAb I diluted to 2 µg/mL in PBS by adding 100 µL/well. Incubate over night at 4-8°C.
- Wash twice with PBS (200 µL/well)
- Block plate by adding 200 µL/well of incubation buffer. Incubate for 1h at room temperature.

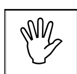
- Wash five times with PBS-Tween (200 µL/well)
- Prepare standard as stated in the manufacturers protocol (differs between cytokines).
- Add 100µL/well of samples or standards diluted in incubation buffer and incubate for 2h at room temperature.
- Wash five times with PBS-Tween (200 µL/well).
- Add 100 µL/well of mAb II at concentration stated in the manufacturers protocol. Incubate for 1h at room temperature.
- Wash five times with PBS-Tween (200 µL/well).
- Add 100 µL/well of Streptadivin-ALP diluted 1:1000 in incubation buffer. Incubate for 1h at room temperature.
- Wash five times with PBS-Tween (200 µL/well).
- Add 100 µL/well of appropriate substrate solution (p-nitrophenyl-phosphate, pNPP). Dissolve 1 tablet of pNPP in 5 mL substrate solution.
- Measure the optical density (405 nm for pNPP) in an ELISA reader after around 5 minutes (higher standard concentrations should be yellowish).

3.3.12.6.2 Jurkat T cells

- Expose cells to nanoparticles (1.5 x 10^5 cells in 12 well plates in a volume of 1 ml for 24h)
- Harvest supernatants from cells exposed to nanoparticles by spinning down the cell culture plate for 3 minutes at 3000 rpm.
- Store supernatants in case needed at -70°C, preferably in small aliquots.
- Coat ELISA plates with 100µl/well of capture antibody in coating buffer. Incubate over night at 4°C.
- Wash 5x with wash buffer (250 µl/well)
- Block plate by adding 200 µl/well of assay diluent. Incubate for 1h at room temperature.
- Wash 5 x with wash buffer (250 µl/well)
- Prepare standard as stated in the manufacturers protocol.
- Add 100µl/well of samples or standards and incubate for 2h at room temperature.
- Wash 5 x with wash buffer (250 µl/well)
- Add 100 µL/well of detection antibody diluted in assay diluent. Incubate for 1h at room temperature.
- Wash 5 x with wash buffer (250 µl/well).
- Add 100 µL/well of Avidin-HRP diluted in assay diluent. Incubate for 30 min at room temperature.

- Wash 7 x with wash buffer (250 µl/well).
- Add 100 µl/well of substrate solution and incubate plate at room temperature for 15 min.
- Add 50 µl of stop solution to each well
- Read plate at 450 nm

3.3.12.7 Control for assay-nanoparticle interactions



In general, nanoparticle interference in this test is relatively low as centrifugated supernatants of exposed cells are used for this test. As a possible control, one sample can be done with the higest nanoparticle concentration used in the assay without cells. This well will be treated similar to the experimental samples. From this sample, no signal should be obtained in the ELISA unless the nanoparticles to exclude interference of nanoparticles with reagents of the ELISA kit.

3.3.12.8 References

(1) Vallhov H, Qin J, Johansson SM, Ahlborg N, Muhammed MA, Scheynius A, Gabrielsson S. Mesoporous silica particles induce size dependent effects on human dendritic cells. Nano Lett. 2007; 7:3576-82.

3.3.13 SOP NANOMMUNE 3.25 Cytokine measurement by ELISpot assay

(Partner 1a: Erika Witasp)

3.3.13.1 Purpose

To determine cytokine release from human monocyte derived macrophages after exposure to nanoparticles.

3.3.13.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the determination of cytokine release from macrophages after exposure to nanoparticles.

3.3.13.3 Principle

The Enzyme-Linked ImmunoSpot (ELISpot) assay is one of the more sensitive immunoassay. available that allow the detection of a secreted cytokine at the single cell level (http://www.mabtech.com). It has been previously reported that nanomaterials could interfere with classical in vitro techniques and ELISpot present an alternative method to detect possible cytokine response of macrophages after exposure of nanoparticles. The ELISpot assay could be summarized in few basic steps: 1) coat the plate with cytokine-specific monoclonal antibodies; 2) add cells with or without stimuli (nanoparticles); 3) add the detection anti-cytokine antibodies to the wells; 4) add the enzyme conjugated Streptavidin; 5) add the substrate that will react with the enzyme to form a colored precipitate; then the spots formed could be counted with an automated ELISpot reader

3.3.13.4 Reagents and Materials

- ELISpot kit for cytokine of interest (MIP-1β, GM-CSF,.TNFα, IL-1, IL-10, IL-12 IL-12,) (Mabtech AB)
- Phosphate buffered saline (PBS) pH 7.4 sterile
- Sterile H_20.
- PBS with 0.5% FCS (PBS - Fetal Calf Serum)
- ELISpot plates.(MAIPSWU10 from Millipore, Billerica, MA)
- BCIP/NBT substrate solution (Mabtech AB)
- Disposable plastic pipettes
- Pipette aid

3.3.13.5 *Safety procedures and precautions*

- Standard safety operating procedures are to be followed at all times.
- Treat all material as infectious.
- All procedures (unless otherwise stated) are to be performed in a Biohazard Class IISafety Cabinet.
- Lab coats, nitril gloves or double vinyl or latex gloves must be worn at all times.

3.3.13.6 *Procedure*

3.3.13.6.1 ELISpot coating protocol for plate (MAIPSWU10 from Millipore, Billerica, MA)

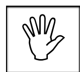
- Add 50µl per well of 70% ethanol for 1 min.
- Discard the alcohol and wash the plate 5x with 200µl per well of sterile H_20.
- Leave the plate filled with sterile H_20 and begin to prepare the coating antibodies
  - Dilute antibodies in PBS to 15µg/ml and add 100µl/well. Note that the membrane should not be dried.
- Incubate the plate overnight in the fridge at 4ºC.

3.3.13.6.2 Protocoll for ELISpot.

- Discard unbound antibodies by washing the plate 5x with 200µl/well sterile PBS.
- Block the plate for 30min by adding 200µl/well of cell culture medium containing 10% FCS
- Discard the medium and add 50 µl per well of fresh cell culture medium with or without stimuli. Do not allow the membrane to dry out.
- Prepare the cells and add them in desired concentrations to the ELISpot wells. Note that both cells and stimuli gets diluted 2x in the well.
- Wrap the plate in aluminum foil and incubate overnight in 37°C 5%CO_2
- When the experiment is done discard the cells and wash plate 5x using 200µl/well PBS
- Dilute all detection antibodies to 1µg/ml in PBS containing 0.5% FCS and add 100µl of the antibody solution to each well. Incubate for 2h at room temperature.
- Wash plate 5x using 200 µl PBS.
- Dilute the Streptavidin-ALP to 1µg/ml in PBS containing 0.5% FCS and add 100µl to each well. Incubate for 1h at room temperature.
- Wash plate 5x using 200 µl PBS.
- Add 100µl/well of freshly filtered (0.45µm) substrate in order to avoid false spots may appear and develop for 8-9min at room temperature. Stop the reaction by washing the plates extensively under tap-water.

- The spots formed could be counted with an automated ELISpot reader.

3.3.13.6.3 Interference assay



In order to evaluate whether nanoparticles interfere with the ELISpot assay, interference assay should be perform. For that reason, cells should be added to each well depending on the cytokine analysis and co-incubated with or without nanoparticles at desired concentration in the presence of 0.1 µg/ml lipopolysaccharide (LPS), then the spots formed could be counted with an automated ELISpot reader and compared. For instance, LPS will induce a significant release of TNF α, a pro-inflammatory cytokine known to be released from activated HMDM (Witasp et al 2009).

3.3.13.7 References

(1) Witasp E, Kupferschmidt N, Bengtsson L, Hultenby K, Smedman C, Paulie S, Garcia-Bennett AE, Fadeel B. Efficient internalization of mesoporous silica particles of different sizes by primary human macrophages without impairment of macrophage clearance of apoptotic or antibody-opsonized target cells. Toxicol Appl Pharmacol. 2009 15;239(3):306-19.

(2) Smedman C, Gårdlund B, Nihlmark K, Gille-Johnson P, Andersson J, Paulie S. ELISpot analysis of LPS-stimulated leukocytes: human granulocytes selectively secrete IL-8, MIP-1beta and TNF-alpha. J Immunol Methods. 2009 31;346(1-2):1-8.

(3) http://www.mabtech.com

3.3.14 SOP NANOMMUNE 3.28 Cytokine measurement using xMAP™ Technology (Luminex)

(Partner 10a: Nancy A. Monteiro-Riviere)

3.3.14.1 Purpose

To determine cytokine release from human cell lines following exposure to nanoparticles.

3.3.14.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the determination of cytokine release from human cell lines following exposure to nanoparticles.

3.3.14.3 3. Principle

Human cytokines from cell culture media can be quantitated by utilizing the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). The system multiplexes to simultaneously assay multiple cytokines using only 50µl of cell culture medium. Magnetic beads conjugated to a capture antibody specific to each cytokine of interest possess a unique spectral address and are incubated in a 96-well filter plate with each media sample. The beads are then incubated with a fluorescent-labeled reporter molecule that specifically binds the analyte. The contents of each well are analyzed in the Bio-Plex array reader (Luminex *x*MAP™ Technology). To accomplish this, the beads are siphoned into the reader and the fluorescent coded spectral address of each cytokine and the reporter molecule excited by a laser. Each specific cytokine is quantitated relative to a standard curve.

3.3.14.4 Reagents and Materials

- Bio-Plex kit for cytokine of interest. Refer to Bio-Rad Laboratories website (http://www.bio-rad.com/evportal/en/US/evolutionPortal.portal?_nfpb=true&_pageLabel =productsPage&catID=b30acf0c-b9fc-458c-9f88-9414e9dfa9f7
- Go to link to configure and price kits: www.bio-rad.com/bio-plex/x-plex
- Bio-Plex Instrument
- Vacuum manifold or Bio-Plex Wash Station
- Plate shaker
- Pipetters – Single channel (1000µl and 200µl) and multichannel (200µl)
- Aluminum foil

3.3.14.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Lab coats, nitrile gloves or double vinyl or latex gloves must be worn at all times.
- Treat all culture medium from human cells as a potential biohazard.

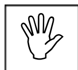
3.3.14.6 Procedure

- Plate cells at appropriate concentration in 200µl of medium in the inner wells of a 96-well plate for 24h. The outer wells of the plate are filled with media or Hanks' Balanced Salt Solution (HBSS) to help minimize evaporation of the medium on the cells.
- Expose cells to nanoparticles, negative controls, and positive controls relevant to the treatment protocol at 37°C under cell culture conditions.
- Carefully aspirate treatment medium, pool by treatment, aliquot, and store at -80°C for later cytokine analysis. At this point, a viability assay is run on the cells.
- Assay is performed using the kits noted above. Determine Sample (in duplicate or triplicate), Standard (in duplicate; total of 16 wells), and Blank (2 wells) layout.
- Cell culture media is removed from the freezer and allowed to melt over several hours in the refrigerator. Media should be routinely centrifuged in microcentrifuge 5 minutes to pellet cell debris and nanoparticles.
- Add BSA to a final concentration of 0.5% to all samples and culture medium used in the assay. Adding the BSA to culture medium blocks nonspecific binding of antibodies, and according to Bio-Rad tech support significantly improves sensitivity of the assay.
- Turn on the three units of the Bio-Plex system two hours prior to plate reading. Bring all buffers and diluents to room temperature and place other reagents on ice. Open Bio-Plex Manager 6.0, turn on laser, and perform Start-Up protocol by following Quick Guide menu on the right of the screen.
- Once the laser has warmed (30 minutes), perform Calibration at low RP1 (low PMT) and, if necessary, perform Validation (due every 31 days). Defaults for Bio-Plex Manager 6.0 are set for magnetic beads.
- Adjust vacuum manifold. Turn on house vacuum, place empty 96-well plate in manifold, and adjust vacuum to 1-3 inches Hg. Set up Protocol specific for the target cytokine(s) in the Bio-Plex software. Lot specific protocols are available for download at: www.biorad.com/bio-plex/standards.
- Reconstitute Standard stock in cell culture media (plus BSA carrier) used to culture the cells. Tap down lyophilized cytokine standard vial and add 500µl of culture medium

containing 0.5% BSA to the vial for stock (S1 concentration listed on package insert used for high range standard curve). Flick gently and place on ice for at least 30 min (and no more than 4 hours) to completely dissolve.

- Prepare serial dilutions for Standard Curve from S1 stock using cell culture media plus 0.5% BSA. Prepare serial dilutions 1:4 as outlined on "Standard Curve" page.
- Prepare the magnetic beads and store on ice. Vortex the anti-cytokine bead stock solution at medium speed for 30 seconds.
- Begin the assay. Pre-wet the filter plate with 100µl/well of Bio-Plex Assay Buffer, vacuum filter with vacuum manifold, and blot dry. While it is important that all the liquid be removed from each well, over filtering may damage the filter membrane and cause leakage.
- Vortex the working Bead solution at medium speed for 30 seconds. Place 50µl in each well of the plate with multi-well pipette. Vacuum filter, wash 2x with 100µl per well of the Bio-Plex Wash Buffer, and blot dry.
- Vortex each Standard for 5 seconds and add 50µl to the appropriate well. Add 50µl.of prepared samples to appropriate wells. Add 50µl cell culture media to the blanks. Cover the plate with the adhesive plate sealer, cover with aluminum foil to occlude light, and incubate on plate shaker for 30 minutes (shake at 1,100 rpm for 30 seconds to suspend beads, then reduce to 300 rpm for remainder of incubation).
- Prepare Detection Antibody and store on ice. Remove plate sealer, filter, wash 3x with 100µl per well of the Bio-Plex Wash Buffer, and blot dry. Vortex the Detection Antibody and add 25µl to each well of the plate. Cover and incubate 30 min on plate shaker as above.
- Prepare SA-PE Antibody and store on ice. Remove plate sealer, filter, wash 3x with 100µl per well of the Bio-Plex Wash Buffer and blot dry. Vortex the SA-PE working solution and add 50µl to each well of the plate. Cover and incubate 10 minutes on plate shaker as above.
- Remove plate sealer, filter, wash 3x with 100µl per well of the Bio-Plex Wash Buffer, and blot dry. Resuspend the beads in each well with 125µl of Bio-Plex Assay Buffer. Cover and shake at 1,100 rpm for 30 seconds to suspend beads.
- Remove plate sealer, remove plate from incubation tray, and place in retractable tray of the Luminex. Click OK and you will be prompted to name Results file. Click OK and the plate read begins.
- Once plate is run, remove from tray, cover with plate sealer and aluminum, and store in refrigerator. Can be rerun within 48 hours by filtering and resuspending beads in each well with 125µl of Bio-Plex Assay Buffer.

- Check the results of the standard curve. Remove any outliers to tighten curve, export data to Excel spreadsheet, and calculate the means. The data can be normalized by the respective viability data to report cytokine release relative to living cells in each treatment.
- Spiked controls should be included with each experiment to determine whether the nanoparticles affect the cytokine assay. To accomplish this, stock cytokine is mixed with the nanoparticles to provide a specific nanoparticle concentration in a known cytokine concentration. Sample(s) should be incubated for the same amount of time as the cell exposure to the nanoparticles (24h). Any significant difference between the theoretical and the actual cytokine values indicates nanoparticle interaction with the assay.



3.3.14.7 References

(1) Ryman-Rasmussen JP, Riviere JE, Monteiro-Riviere NA: Surface coatings determine cytotoxicity and irritation potential of quantum dot nanoparticles in epidermal keratinocytesJournal of Investigative Dermatology. 127:143-153, 2007.
(2) Zhang LW, Zeng L, Barron AR, Monteiro-Riviere NA:.Biological interactions of functionalized single-wall carbon nanotubes in human epidermal keratinocytesInternational Journal of Toxicology. 26:103-113, 2007.
(3) Rouse JG, Yang J, Barron AR, Monteiro-Riviere NA:.Fullerene-based amino acid nanoparticle interactions with human epidermal keratinocytesToxicology In Vitro 20: 1313-1320, 2006.
(4) Samberg ME, Oldenburg SJ, Monteiro-Riviere NA. Evaluation of silver nanoparticle toxicity in vivo skin and in vitro keratinocytes. Environmental Health Perspectives 118, 407-413, 2010.
(5) Monteiro-Riviere NA, Oldenburg SJ, Inman AO. Interactions of aluminum nanoparticles with human epidermal keratinocytes. Journal of Applied.Toxicology_30:276-285, 2010.

3.3.15 SOP NANOMMUNE 3.22 v1 Detection of intracellular free Zinc (II) by Zinquin

(Partner 6: Tina Bürki)

3.3.15.1 Purpose

To detect the presence of intracellular Zinc-ions in cells exposed to zinc nanoparticles.

3.3.15.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the detection of intracellular Zinc-ions in cells exposed to zinc nanoparticles.

3.3.15.3 Principle

Zinquin ethyl ester is a lipophilic, zinc-sensitive, fluorescent derivative of Zinquin that is able to penetrate cell membranes. Cleavage of the ethyl ester group via cytosolic esterases in living cells impedes its efflux across the plasma membrane. It is selective for Zn (II) in the presence of physiological concentrations of Ca (II) and Mg (II) ions. Fluorescence of Zinquin ethyl ester is enhanced upon binding Zn (II), but the probe does not demonstrate a shift or change in emission wavelength. The probe is UV-excitable and emits in the blue reqion of the spectrum. Zinquin ethyl ester has been used to monitor intracellular zinc fluxes associated with apoptosis.
Wavelength Maxima: Excitation 368nm, Emission 490nm.
Zinc-salts (e.g. ZnCl2, ZnSO4) can be used as positive control.

3.3.15.4 Reagents

- Zinquin (Alexis/Enzo, 620-044-M001); stock solution 2.4mM in DMSO (1mg/ml)
- 1x PBS (176.8 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4,8.1 mM Na_2HPO_4 x $2H_2O$)
- HBSS (Hank's buffered salt solution) (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4, 4.2 mM NaHCO3)
- 4% Paraformaldehyde (PFA) (Riedel de-Haen, 16005) in PBS
- Poly-D-lysine (PDL) (Sigma, P7280)
- Mowiol (Sigma; 81381)

3.3.15.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
- Follow safety guidelines provided by the reagent and material manufacturer at all times.

3.3.15.6 Procedure

- Incubate cells with different concentrations of nanoparticles for the desired time (e.g. 1.5 x 10^5 Jurkat cells in 24 well plates in 1ml)
- Suggested controls: Equimolar concentrations of ZnCl2
- Collect the cells in a centrifugation tube and centrifuge for 3 min at 4000 rpm
- Wash pellet with HBSS
- Add 500 µl of 25 µM Zinquin and incubate for 30 min at 37°C
- Collect the cells in a centrifugation tube and centrifuge for 3 min at 4000 rpm
- Wash 2x with HBSS
- Resuspend pellet a small volume of HBSS (e.g. 100 µl; has to be adjusted for each cell type and condition in order to obtain a reasonable cell density on the slide)
- Carefully add a drop (e.g. 50 µl) of the cell suspension on a round glass slide coated with 25 µg/ml PDL (add glass slides into wells of a 24 well plate and coat with 500 µl 25 µg/ml PDL for 15 min, then wash 2x with PBS; prepare in advance!)
- Let cells adhere for around 5-10 min (check if cells attached to slide by gentle shaking; to avoid that the drop leaves the glass slide and cells are sucked below the glass and to the wells, the glas slides should be dry or they can be placed onto a prarfilm)
- Fix cells with 4% PFA for 10 min at room temperature
- Wash with PBS
- At this step, additional staining can be done (e.g. nuclear staining)
- Mount slides using Mowiol for longer conservation or microscope them directly

3.3.16 SOP NANOMMUNE 3.10v1 TEM analysis of cells after exposure to nanoparticles

(Partner 6: Tina Bürki)

3.3.16.1 Purpose

To prepare cells after exposure to nanoparticles for EM analysis.

3.3.16.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the shipment and embedding of nanoparticle-treated cells for TEM analysis.

3.3.16.3 Principle

As nanoparticles are very small, transmission electron microscopy is required to study the interaction of nanoparticles with cells (e.g. uptake, localization). The TEM operates on the same principles as the light microscope but uses a beam of electrons instead of light. As the wavelength of electrons is much lower, it is possible to get a resolution a thousand times better than with a light microscope.

3.3.16.4 Reagents

- Cellulose microcapillary tubes (Leica 16706869)
- Fixation buffer

  0.2 M sodium cacodylate buffer, pH7.4

  measure out 42.8 g sodium cacodylate trihydrate (Fluka 20840, $C_2H_6AsNaO_2{\bullet}3H_2O$ Mr214.03)

  add 1000 ml of ultrapure water (MilliQ, Millipore), mix well with magnetic stirrer

  adjust pH to 7.4
- Store at 4°C

  Prepare fresh!!
- Fixation buffer: 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH7.4

  mix equal volumes of 6% glutaraldehyde stock and 0.2 M sodium cacodylate buffer pH7.4

  Prepare freshly
- 4% osmium tetroxide (Johnson Matthey, 7.2002, osmium (VIII) oxid solid), store at -20°C
- Epon resin (Fluka)
- 2% uranyl acetate, store at 4°C (Fluka)
- Lead citrate (Reynolds 1963)

3.3.16.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times
- Lab coats, safety glasses, nitril gloves or double vinyl or latex gloves must be worn all times
- All procedures are to be performed in a safety cabinet
- Special disposal is required as many substances are highly toxic (e.g. osmium tetroxide, uranyl acetate, lead citrate)

3.3.16.6 Procedure

- Incubate the cells with different concentrations of nanoparticles for the desired time
- Collect the cells in a centrifugation tube (adherent cells have to be trypsinized)
- Wash cells with cold PBS
- Resuspend cells in fixation buffer and store at 4°C; at this step, cells can be shipped at 4°C to another lab for TEM embedding and analysis (see 7.1.-7.4.)
- Suck up cells into a cellulose capillary tube
- Rinse cells in 0.2 M sodium cacodylate buffer pH 7.4
- Postfix cells in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.4 at 4°C for 30 min
- Dehydrate through a graded ethanol series followed by acetone
- Embed cells in Epon resin
- Cut ultrathin sections and contrast with 2% uranyl acetate and lead citrate 1

3.3.16.7 Sending of samples

- Fixed cells can be shipped to another lab for TEM embedding and analysis (see 6.4.)
- Shipment should be within 1-2 days; if possible send at least 1.5 x 106 cells; for experiments where cell number is limited try to send 0.5 x 106 cells
- Send fixed cells in a tightly closed and parafilm-sealed Eppendorf tube at 4°C (use cooling elements) in a styrofoam box

3.3.16.8 References

(1) Reynolds ES. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of cell biology 17: 208-212

3.3.17 SOP NANOMMUNE 3.30 Transmission electron microscopy (TEM) analysis of cells following exposure to nanoparticles

(Partner 10a: Nancy A. Monteiro-Riviere)

3.3.17.1 Purpose

To prepare cells after exposure to nanoparticles for TEM analysis.

3.3.17.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the preparation, processing, and embedding of nanoparticle-treated cells for TEM analysis.

3.3.17.3 Principle

Since nanoparticles are typically around 100nm in size, transmission electron microscopy is required to study the interaction of these particles with cells (e.g. uptake, localization). The TEM operates on the same principle as the light microscope, but uses a beam of electrons rather than visible light to visualize the sample. Due to the extremely short wavelength of electrons, the resolution a thousand times better than that of the light microscope.

3.3.17.4 Reagents

- 3% agar. Made up in distilled water and store in 50ml centrifuge tube at 4°C.
- Sodium phosphate buffer, 0.2M
- Stock A: 0.2M sodium phosphate monobasic (1 H_2O). 27.6g/L in distilled water
- Stock B: 0.2M sodium phosphate dibasic (anhydrous). 28.4g/L in distilled water
- Add 28.0ml of Stock A to 72.0ml of Stock B for a 0.2M solution, pH to 7.2.Store at 4°C.

- Trumps' Fixative
- Mix 88ml distilled water, 10ml formaldehyde (37%), 2ml glutaraldehyde (50%, EM grade), 1.16g sodium phosphate monobasic (1 H_2O), and 0.27g of sodium hydroxide. Adjust pH to 7.2. Store at 4°C.
- Fixative is stable for 3 months at 4°C. Tissues can be stored for up to one year at 4°C.
- Osmium tetroxide. Stock solution

4% stock solution. Remove ampoule containing 1g of osmium (Polysciences, Inc; 0223B) from refrigerator and place under hot water to melt osmium crystals. Rotate under cold water to create a thin layer of osmium on the walls of the ampoule. Pop the pre-scored top

off the ampoule and drop in an amber glass bottle containing 50ml of ultrapure water. Cap the bottle and place in a light-tight secondary container and allow osmium to dissolve overnight. Parafilm containers and store at 4°C.

All glassware, including osmium ampoule, must be extremely clean. Rinse in water, and then rinse in 95% ethanol to remove any residue from the glass. Rinse thoroughly with distilled water, and allow to air. Wear gloves and work under hood. The osmium vapors are extremely corrosive to delicate tissues of the eye and the nose.

- Spurr resin (Ted Pella, Inc; 18300-4221). Weigh in clean bottle.

  ERL-4221, 10g; D.E.R. 736 Epoxy Resin, 6.3g; Nonenyl Succinic Anhydride, 26g; DMAE, 0.4g.Mix thoroughly. Unused portion can be stored up to 2 weeks at 4°C.

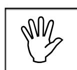
3.3.17.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times
- Lab coats, safety glasses, nitrile gloves must be worn all times
- All procedures are to be performed in a chemical hood.
- Hazardous waste disposal is required as many substances are highly toxic (e.g. Trump's, fixative, osmium tetroxide, ethanols)

3.3.17.6 Procedure

- Incubate the cells with the highest concentration of nanoparticles that provides at least 50% viability in T-25 cell culture flasks.
- Trypsinize cells per protocol, rinse in Hanks' Balanced Salt Solution (HBSS), aspirate rinse, and resuspend cells in ~2ml of Trump's fixative at 4°C for a minimum of 24h.
- Following fixation, centrifuge cells to a pellet, carefully aspirate fixative, and resuspend in ~3ml of 0.1M phosphate buffer to rinse. Centrifuge to pellet, resuspend in 1ml of phosphate buffer, and transfer cells to 1.5ml microfuge tube.
- Melt 3% agar by placing tube in boiling water bath. Centrifuge cells to pellet, remove buffer, resuspend cells in ~200µl molten agar, and quickly pulse spin to pellet. Cool agar by dipping in cold water, cut the tip of the microfuge tube containing agar-embedded cells, and place in cold 0.1M phosphate buffer.
- Postfix cells in 2% osmium tetroxide in 0.1 M sodium phosphate buffer 4°C for 60 min.
- Dehydrate through a graded ethanol series (50%, 70%, and 95% for 10 min; 100% 3 x 10 min), followed by 2 x 10 min acetone clear.

- Infiltrate cells in 50% Spurr resin (in acetone) for 30 min and 2 x 60 min pure resin. Embed cells in Spurr resin and cure overnight at 70°C.
- Cut ultrathin sections at ~800Å with a diamond knife and mount on carbon-coated copper grids (for possible EDS analysis). Do not stain with 2% uranyl acetate and lead citrate for visualization of nanoparticles. The staining makes finding the nanoparticles more difficult and stain precipitant can be mistaken for nanoparticles of interest. In addition, the heavy metal stains add additional peaks to an EDS spectrum.
- Alternate method for imaging nanoparticles within cell monolayer. Incubate the cells with the highest concentration of nanoparticles that provides at least 50% viability in sterile Permanox-coated culture dishes (Electron Microscopy Sciences; cat# 70340). Rinse, fix, process, infiltrate, and embed cells in the dish as above, since the Permanox protects the dish during the acetone and Spurr steps. Once Spurr has polymerized overnight at 70°C, the cells are removed from the dish with the resin. Cut the resin into $1mm^2$ pieces, embed in a flat mold, and cure overnight.



3.3.17.7 References

(1) Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY, Riviere JE:.Multi-walled carbon nanotube interactions with human epidermal keratinocytesToxicol. Lett. 2005; 155: 377-384

4 In Vivo Toxicity Testing

4.1 Characterisation of lung samples

4.1.1 SOP NANOMMUNE 4.01v1 Preparation of lung homogenates

(Partner 9: Anna Shvedova)

4.1.1.1 Purpose

To prepare lung homogenates to be used to measure toxicity after exposure to nanoparticles.

4.1.1.2 Scope

This protocol provides a descriptive procedure detailing the preparation of lung homogenates for the use of measuring toxicity following exposure to nanoparticles.

4.1.1.3 Principle

This method of tissue processing is used for the quantitative analysis of lung samples taken from mice after exposure to nanoparticles. Homogenization of the tissue yields a uniform semisolid that can be analyzed for amount of total protein, protein carbonyls and antioxidant status as evidence of toxicity.

4.1.1.4 Reagents

- PBS

4.1.1.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times. Avoid inhalation. Avoid contact with eyes, skin and clothing.
- Follow safety guidelines provided by the manufacturer at all times.

4.1.1.6 Procedure

- Whole mouse lungs were separated from other tissues and weighed.
- 700µl of PBS was added to each whole lung.
- A tissue tearer (model 985-370, Biospec Products Inc., Racine, WI) was used to homogenate the lung tissue for 2 minutes.
- The homogenates were stored at -80°C until processed.

4.1.1.7 References

(1) Yu C. and Cohen L. (2004). Tissue sample preparation-not the same old grind. Pfizer Global Research and Development, Ann Arbor, Michigan, USA.

4.1.2 SOP NANOMMUNE 4.02v1
Total protein level in lung homogenates
(Partner 9: Anna Shvedova)

4.1.2.1 Purpose

To measure total protein in lung homogenates as a measure of pulmonary damage after exposure to nanoparticles.

4.1.2.2 Scope

These protocols provide descriptive procedures detailing the detection of total protein as a measure of in vivo toxicity after exposure to nanoparticles.

4.1.2.3 Principle

The modified Bradford assay is a colorimetric assay that measures the color change of a dye when it binds to protein and can be used to quantitatively measure total protein concentration. The absorbance maximum of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when it binds to primarily basic and aromatic amino acid residues. Based on the observation that the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range, Beer's law can be applied to determine concentration of protein when an appropriate ratio of dye volume to sample concentration is used. Increased levels of total protein in BAL are an indication of inflammatory response.

4.1.2.4 Reagents

- Protein Assay Dye Reagent Concentrate (Biorad, Hercules, CA)
- Bovine serum albumin standard

4.1.2.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
- Follow safety guidelines provided by the reagent and material manufacturer at all times.

4.1.2.6 Procedure

- Prepare bovine serum albumin standard curve dilutions: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml.
- Dilute dye reagent 1:4 in distilled water.

- Pipet 10 µl of each standard and sample (sample are run in quintuplicates) into a 96-well plate.
- Add 190 µl dye reagent.
- Incubate for 5 minutes.
- Measure the absorption at 595 nm in spectrophotometer.
- Determine the average values from quintuplicate readings.
- Create a standard curve by plotting absorbance on the y-axis and concentration on the x-axis using the standards.
- Calculate protein concentrations from the average absorbance readings from the standard curve.

4.1.2.7 References

(1) Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72: 248-254.

(2) Spector T. Refinement of the Coomassie Blue Method of Protein Quantitation. Anal. Biochem. 1978; 86: 142-146.

4.1.3 SOP NANOMMUNE 4.03v1
Total protein and lactate dehydrogenase (LDH) activity in BAL fluid
(Partner 9: Anna Shvedova)

4.1.3.1 Purpose

To measure total protein and lactate dehydrogenase activity in BAL fluid as a measure of pulmonary damage after exposure to nanoparticles.

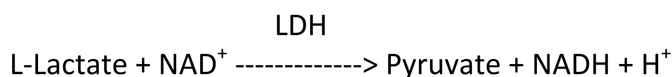
4.1.3.2 Scope

These protocols provide descriptive procedures detailing the detection of total protein and lactate dehydrogenase activity as a measure of in vivo toxicity after exposure to nanoparticles.

4.1.3.3 Principle

The modified Bradford assay is a colorimetric assay that measures the color change of a dye when it binds to protein and can be used to quantitatively measure total protein concentration. The absorbance maximum of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when it binds to primarily basic and aromatic amino acid residues. Based on the observation that the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range, Beer's law can be applied to determine concentration of protein when an appropriate ratio of dye volume to sample concentration is used. Increased levels of total protein in BAL are an indication of inflammatory response.

Lactate dehydrogenase catalyzes the oxidation of lactate to pyruvate. During this reaction NAD is reduced to NADH.



This assay measures the rate of NAD reduction as an increase in absorbance at 340 nm which is directly proportional to LDH activity in serum. LDH is a soluble enzyme located in the cytosol; when cell damage occurs it is released. The presence of LDH activity is an indicator of cytoxicity.

4.1.3.4 Reagents

Total protein measurement

- Protein Assay Dye Reagent Concentrate (Biorad, Hercules, CA)

- Bovine serum albumin standard

Reagents: LDH activity

- Lactate dehydrogenase buffer reagent (Pointe Scientific, Inc., Lincoln Park, MI)
- Lactate dehydrogenase co-enzyme reagent (Pointe Scientific, Inc., Lincoln Park, MI)

4.1.3.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
- Follow safety guidelines provided by the reagent and material manufacturer at all times.

4.1.3.6 Procedure

Total protein measurement

- Prepare bovine serum albumin standard curve dilutions: 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml.
- Dilute dye reagent 1:4 in distilled water.
- Pipet 10 µl of each standard and sample (sample are run in quintuplicates) into a 96-well plate.
- Add 190 µl dye reagent.
- Incubate for 5 minutes.
- Measure the absorption at 595 nm in spectrophotometer.
- Determine the average values from quintuplicate readings.
- Create a standard curve by plotting absorbance on the y-axis and concentration on the x-axis using the standards.
- Calculate protein concentrations from the average absorbance readings from the standard curve.

Procedure: LDH activity

- Prepare working reagent mix 5 parts buffer reagent with 1 part co-enzyme reagent.
- Add 1.0 ml of reagent into appropriate tubes and warm for five minutes at 37 °C.
- Add 50 µl of sample to reagent, vortex. Return to 37 °C for 30 seconds.
- Measure absorbance (A_1) at 340 nm.
- After one minute, measure absorbance (A_2) at 340 nm.
- Multiply A_2-A_1 by the factor 3376 to calculate results in U/L. One U/L is the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute.

4.1.3.7 References

(1) Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72: 248-254.

(2) Spector T. Refinement of the Coomassie Blue Method of Protein Quantitation. Anal. Biochem. 1978; 86: 142-146.

(3) Henderson R. Benson J. Hahn F. Hobbs C. Jones R. Mauderly J. McClelian R. Pickrell J. New Approaches for the Evaluation of Pulmonary Toxicity: Bronchoalveolar Lavage Fluid Analysis. Fund. Appl. Toxicol. 1985; 5: 451-458.

(4) Henderson R. Damon E. Henderson T. Early damage indicators in the lung l. lactate dehydrogenase activity in the airways. Toxicol. Appl. Pharmacol. 1977; 44: 291-297.

(5) Haslam G. Wyatt D. Kitos. PA. Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. Cytotechnology 2000; 36: 63-75.

4.1.4 SOP NANOMMUNE 4.04v1 Lung Collagen Measurements
(Partner 9: Anna Shvedova)

4.1.4.1 Purpose
To measure total soluble lung collagen as a measure of pulmonary damage after exposure to nanoparticles.

4.1.4.2 Scope
These protocols provide descriptive procedures detailing the detection of total soluble lung collagen as a measure of in vivo toxicity after exposure to nanoparticles.

4.1.4.3 Principle
In this assay Sirius Red dye binds to collagen and the amount of collagen present in a sample is proportional to the absorbance at 555 nm. The assay can assess the rate of newly synthesized collagen generated during processes such as inflammation in the lung.

4.1.4.4 Reagents
- Sircol Soluble Collagen Assay kit (Accurate Chemical and Scientific Corporation, Westbury, NY)
- Dye reagent: Sirius Red in picric acid
- 0.5 M sodium hydroxide
- Sterile solution of cold acid-soluble collagen Type I, in 0.5 M acetic acid
- Acid-salt was reagent: acetic acid, sodium chloride and surfactants
- Acid neutralizing reagent: TRIS-HCl and NaOH
- Collagen isolation and concentration reagent: polyethylene glycol in TRIS-HCl buffer
- 0.5 M acetic acid
- Pepsin

4.1.4.5 Safety procedures and precautions
- Standard safety operation procedures are to be followed at all times.
  Avoid inhalation. Avoid contact with eyes, skin and clothing.
- Follow safety guidelines provided by the manufacturer at all times.

4.1.4.6 *Procedure*

- Homogenize whole lungs in 0.7 ml of 0.5 M acetic acid containing pepsin with 1:10 ratio of pepsin/tissue wet weight.
- Stir each sample vigorously for 24 hours at 4°C and then centrifuge.
- Prepare reagent blanks, collagen standards and test samples: 200 µl per microcentrifuge tube.
- Add 1.0 ml Sircol Dye reagent to each tube and place on a mechanical shaker for 30 minutes.
- Centrifuge at 12,000 rpm for 10 minutes; invert and drain tubes.
- Add 750 µl ice-cold acid-salt wash reagent to each tube.
- Centrifuge at 12,000 rpm for 10 minutes; invert and drain tubes.
- Add 250 µl alkali reagent to reagent blanks, standards and samples.
- Re-suspend pellet and transfer 200 µl of each sample to a 96 well plate.
- Read plate at 555 nm.

4.1.4.7 *References*

(1) Elias J., Freundlich B., Kern J. and Rosenbloom J. Cytokine networks in the regulation of inflammation and fibrosis in the lung. Chest 1990; 97: 1439-1445.

(2) Khalil N., Bereznay O., Sporn M. and Greenberg A. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. J. Exp. Med. 1989; 170: 727-737.

(3) Johnson J., Wiley R., Fattouh R., Swirski F., Gajewska B., Coyle A., Gutierrez-Ramos, J., Ellis R., Inman M. and Jordana M. Continuous exposure to house dust elicits chronic airway inflammation and structural remodeling. Am. J. Respir. Crit. Care Med. 2004; 169: 378-385.

4.1.5 SOP NANOMMUNE 4.05v1 Quantitative measurement of total antioxidant status in lung homogenates

(Partner 9: Anna Shvedova)

4.1.5.1 Purpose

To measure total lung antioxidant status as a measure of pulmonary damage after exposure to nanoparticles.

4.1.5.2 Scope

This protocol provides a descriptive procedure detailing the quantitative measurement of total antioxidant status in lung homogenates following exposure to nanoparticles.

4.1.5.3 Principle

This assay measures the reduction of Cu^{2+} to Cu^{+} by antioxidants in the lung homogenate. Cu^{+} reacts with bathocuproine to form a complex with maximal absorbance at 490 nm. Measuring absorbance before and after the addition of bathocuproine results in a net absorbance proportional to the sample's reductive capacity. An imbalance between reactive oxygen species and total antioxidant capacity is referred to as oxidative stress. This oxidant-antioxidant imbalance is involved in several diseases including chronic inflammatory diseases. Total antioxidants status can be used as an indication of oxidative stress.

4.1.5.4 Reagents

- NWLSS Antioxidant Reductive Capacity assay (Northwest Life Science Specialties, LLC, Vancouver, WA)
- Assay dilution buffer: bathocuprione disulfonic acid
- Cu^{2+} solution
- EDTA stop solution
- Uric acid

4.1.5.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
  Avoid inhalation. Avoid contact with eyes, skin and clothing.
- Follow safety guidelines provided by the manufacturer at all times.

4.1.5.6 Procedure

- Prepare standards: 2.0, 1.0, 0.50, 0.25, 0.125, 0.063 mM.

- Allow samples and standards to come to room temperature.
- Dilute samples and standards 1:40 with assay dilution buffer.
- Add 200 µl of sample or standard to each well.
- Read the plate at 450 nm.
- Add 50 µl of Cu^{2+} solution to each well and incubate 3 minutes at room temperature.
- Add 50 µl of stop solution to each well.
- Read the plate at 450 nm.

4.1.5.7 References

(1) Sies, H. Oxidative stress: oxidant and antioxidants. Academic Press, London, 1991

(2) Vachier I, Damon M, Le Doucen C, Crastesde Paulet-A, Chanez P, Michel FB, et al. Increased oxygen species generation in blood monocytes of asthma patients. Am. Rev. Resp. Dis. 1992; 146: 1161-1166.

(3) Barnes BJ. Reactive oxygen species and airway inflammation. Free Radical Biol. Med. 1990; 9: 235-243.

(4) Nadeem A, Chhabra S, Masood A, Raj H. Increased oxidative stress and altered levels of antioxidants in asthma. J. Allergy Clin. Immunol. 2003; 111: 72-78.

4.1.6 SOP NANOMMUNE 4.06v1 Myeloperoxidase levels in the lung of SWCNT-exposed mice

(Partner 9: Anna Shvedova)

4.1.6.1 Purpose

To test levels of myeloperoxidase as a measure of inflammation after exposure to nanoparticles.

4.1.6.2 Scope

This protocol provides a descriptive procedure detailing the detection of myeloperoxidase as a measure of inflammation after exposure to nanoparticles.

4.1.6.3 Principle

This enzyme-linked immunosorbent assay measures myeloperoxisae (MPO) based on the sandwich principle. MPO is present in azurophilic granules of polymorph nuclear neutrophils (PMN) and catalyzes the conversion of hyrdrogen and chloride to hypochlorous acid, an oxidant with antimicrobial activity.MPO is released by PMNs and is considered to be a marker of inflammation. Lung homogenates are incubated in wells coated with anti- mouse MPO antibody that bind mouse MPO. A biotinylated antibody to mouse MPO is added. Streptavidin peroxidase is added which reacts with the biotinylated antibody bound to MPO. Color develops proportionally to the amount of MPO present and can be measured at 450 nm.

4.1.6.4 Reagents

- Mouse MPO ELISA test kit (Cell Sciences, Canton, MA)
- Wash buffer: Tris buffered saline containing Tween 20
- Dilution buffer A: acetate buffer containing Tween 20
- Dilution buffer B: protein stabilized buffered saline
- MPO standard (750 ng/ml)
- Tracer: biotinylated antibody to mouse MPO in protein stabilized buffer
- Streptavidin peroxidase conjugate
- TMB substrate
- Citric acid stop solution
- ELISA plate coated with anti-mouse MPO antibody

4.1.6.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
  Avoid inhalation. Avoid contact with eyes, skin and clothing.

- Follow safety guidelines provided by the reagent and material manufacturer at all times.

4.1.6.6 Procedure

- Homogenate tissue samples.
- Add 75 µl lysis buffer to 200 µl homogenate and vortex at 4 °C for 15 minutes.
- Prepare dilution series of standard curve and samples.
- Aliquot 100 µl of each standard, sample and control in duplicate into wells of ELISA plate
- Cover and incubate for 1 hour at room temperature.
- Wash wells 4 times with 200 µl wash buffer.
- Add 100 µl diluted tracer (1:11 dilution buffer) to each well.
- Cover and incubate for 1 hour at room temperature.
- Wash wells 4 times with 200 µl wash buffer.
- Add 100 µl of diluted streptavidin-peroxidase conjugate (1:24 dilution buffer) to each well.
- Cover and incubate for 1 hour at room temperature.
- Wash 4 times with 200 µl wash buffer.
- Add 100 µl of TMB substrate solution to each well.
- Cover and incubate for 30 minutes in the dark at room temperature.
- Add 100 µl of stop solution to each well.
- Measure the absorbance at 450 nm.
- Determine the average values from duplicate readings. Plot absorbance on the y-axis versus concentration on the x-axis. Calculate concentration from standard curve and multiply by the dilution factor.

4.1.6.7 References

(1) AE Postlethwaite and AH Kang JI Gallin, IM Goldstein and R Snyderman. Inflammation, basic principles, and clinical correlates, Raven Press, New York (1988), pp. 577–597.

(2) Bradley P. Christensen R. Rothstein G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. J. Am. Soc. Hematol. 1982; 60: 618-622.

4.1.7 SOP NANOMMUNE 4.07v1
Measurement of protein carbonyls in lung homogenates
(Partner 9: Anna Shvedova)

4.1.7.1 Purpose

To assess the quantity of oxidatively modified proteins by measuring protein carbonyls in lung homogenates after exposure to nanoparticles.

4.1.7.2 Scope

This protocol provides a descriptive procedure detailing the measurement of protein carbonyls in lung homogenates as a measure of oxidative injury following exposure to nanoparticles.

4.1.7.3 Principle

This enzyme-linked immunosorbent assay measures protein carbonyls present in BAL fluid. Reactive carbonyl adduction of proteins is a feature of oxidative stress. In this assay the carbonyl groups are reacted with dinitrophenylhydrazine (DNP) which is then bound to the ELISA plate. The DNP-protein is probed with anti-DNP antibody. Streptavidin-linked horseradish peroxidase is bound to the complex. After the addition of chromatin containing peroxide, the change in absorbance at 450 nm is then measured.

4.1.7.4 Reagents

- BIOCELL PC ELISA kit (Northwest Life Sciences Specialties, LLC, Vancouver, WA)
- EIA buffer
- Blocking reagent
- Dinitrophenylhydrazine (DNP)
- Guanidine hydrochloride diluent
- Anti-DNP-biotin-antibody
- Streptavidin-horseradish-peroxidase
- Chromatin reagent
- Stopping reagent
- Serum albumin standards
- Carbonyl control samples

4.1.7.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
  Avoid inhalation. Avoid contact with eyes, skin and clothing.

– Follow safety guidelines provided by the reagent and material manufacturer at all times.

4.1.7.6 *Procedure*

– Add 200 µl of diluted DNP solution to each tube labeled for standards, controls and samples.
– Add 5 µl of each sample, standard or control to the appropriate tube.
– Mix and incubate for 45 minutes.
– In a new set of tubes, add 1 ml EIA buffer to each tube.
– Add 5 µl of each DNP-treated sample to each tube and mix.
– Add 200 µl of each sample into each well of the ELISA plate.
– Cover the plate and leave overnight at 4 °C.
– Wash 5 times with 300 µl EIA buffer.
– Add 250 µl of diluted blocking solution to each well and incubate for 30 minutes at room temperature.
– Wash 5 times with 300 µl EIA buffer.
– Add 200 µl of diluted anti-DNP-biotin-antibody to each well and incubate for 1 hour at 37 °C.
– Wash 5 times with 300 µl EIA buffer.
– Add 200 µl of diluted streptavidin-HRP to each well and incubate for 1 hour at room temperature.
– Wash 5 times with 300 µl EIA buffer.
– Add 200 µl of Chromatin reagent per well and let develop for 4-7 minutes.
– Stop reaction with 100 µl of stopping reagent per well.
– Measure absorbance at 450 nm.
– Determine the average values from duplicate readings. Plot absorbance on the y-axis versus concentration on the x-axis. Calculate concentration from standard curve.

4.1.7.7 *References*

(1) Buss H. Chan T.P. Sluis K.B. Domigan N.M. and Winterbourn C.C. Protein carbonyl measurement by a sensitive ELISA method. Free Rad. Biol. Med. 1997; 23: 361-366.
(2) Winterbourn C.C. and Buss H. Protein carbonyl measurement by enzyme-linked immunosorbent assay. Methods Enzymol. 1999; 300: 106-111.
(3) Dalle-Donne I. Rossi R. Giustarini D. Milzani A. Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 2003; 329: 23-38.

4.1.8 SOP NANOMMUNE 4.03v1 TGF-β1 analysis in BAL fluid

(Partner 9: Anna Shvedova)

4.1.8.1 Purpose

To measure amount of TGF-β1 present in bronchoalveolar lavage fluid after exposure to nanoparticles.

4.1.8.2 Scope

These protocols provide descriptive procedures detailing the detection of TGF-β1 as a measure of in vivo toxicity after exposure to nanoparticles.

4.1.8.3 Principle

An enzyme-linked immunosorbent assay is used to measure TGF-β1 in the BAL fluid. This assay is based on the sandwich principle in which a monoclonal antibody specific for TGF-β1 is coated onto the wells of a plate. The sample and a biotinylated antibody to TGF-β1 is added; streptavidin peroxidase is added next which reacts with the biotinylated antibody bound to TGF-β1. Color develops proportionally to the amount of TGF-β1 present and can be measured at 450 nm. TGF-β1 is an immunoregulatory molecule that has been implicated in many physiologic processes such as inflammation. Here TGF-β1 is measured along with TNF-α, IL-6 and MCP-1 as an indication of pulmonary inflammation after exposure to nanoparticles.

4.1.8.4 Reagents

- ELISA kit (Biosource International Inc., Camarillo, CA)
- TGF-β1 standard, recombinant human TGF-β1
- Standard diluents buffer, 8nM sodium azide
- TGF-β1 anti-body coated 96 well plate
- Extraction solution
- TGF-β1 biotin conjugate
- Streptavidin-peroxidase (HRP)
- Streptavidin-peroxidase (HRP) diluent
- Wash buffer
- Chromogen, tetramethylbenzidine
- Stop solution

4.1.8.5 Safety procedures and precautions

- Standard safety operation procedures are to be followed at all times.
  Avoid inhalation. Avoid contact with eyes, skin and clothing.
- Follow safety guidelines provided by the manufacturer at all times.

4.1.8.6 Procedure

- Add 200µl if standard buffer to zero wells and leave wells for chromogen blank empty.
- Add 200µl of standards, samples and controls to appropriate wells.
- Add 50Al of biotinylated anti0TGF-β1 solution into each well and tap to the plate to mix.
- Cover plate and incubate for 3 hours at room temperature.
- Wash wells four times with wash buffer.
- Add 100µl of Streptavidin-HRP working solution to each well.
- Cover plate and incubate for 3 hours at room temperature.
- Wash wells four times with wash buffer.
- Add 100µl of chromogen to each well.
- Incubate for 30 minutes at room temperature.
- Add 100µl of stop solution to each well.
- Read the absorbance of each well at 450 nm.

4.1.8.7 References

(1) Kim S., Romeo D., Yoo Y. and Park K. Transforming growth factor-beta: expression in normal and pathological conditions. Hormone Res. 1994; 42: 5-8.

(2) Kulkarni A.B. and Karlsson S. Transforming growth factor-beta 1 knockout mice: a mutation in one cytokine gene causes a dramatic inflammatory disease. Am. J. Pathol. 1993; 143: 3-9.

5 Transcriptomics

5.1 Preparation Procedures

5.1.1 SOP NANOMMUNE 5.1v1 Protocol for stabilizing RNA in RNAlater® solution prior to RNA extraction

5.1.1.1 Purpose

The preparation of samples in RNAlater® solution for RNA extraction and microarray analysis by the Turku Center for Biotechnology.

5.1.1.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the preparation of cell culture and tissue samples in RNA stabilizing buffer (RNAlater®, Ambion ®). Further sending and handling of samples is described in protocol NANOMMUNE SOP 5.2.

5.1.1.3 Principle

Storage of cell culture and tissue samples in RNAlater® solution (Ambion ®) is needed to ensure intact RNA for extraction. RNAlater® solution is an aqueous tissue storage reagent that permeates most tissues to stabilize and protect RNA. The cells can simply be submerged in RNAlater® solution and stored for analysis at a later date. Samples in RNAlater® solution can be long term stored (preferably at –20°C or below) without RNA degradation to take place. RNAlater® solution is compatible with most RNA isolation methods.

5.1.1.4 Reagents and materials

- RNAlater® solution, Ambion ®, order number AM7020
- Sterile Phosphate buffered saline pH 7.4
- Micropipette and pipette tips (2-200 µl, 100-1000 µl)
- 1,5-2 ml eppendorf tubes

5.1.1.5 Safety procedures and precautions

- Thoroughly read the manufacturer's instructions for RNAlater® solution
- Standard safety operating procedures are to be followed at all times.
- Treat all human-derived material as infectious.
- Lab coats and latex/vinly/nitril gloves must be worn at all times.

5.1.1.6 Procedure

5.1.1.6.1 General

- Thoroughly read the manufacturer's instructions for RNAlater® solution
- Use RNAlater Solution with fresh tissue only; do not freeze tissues before immersion in RNAlater® solution.
- Place the fresh tissue in 5–10 volumes of RNAlater® solution.
- Freeze samples in RNAlater® solution at –20°C or –80°C before sending.

5.1.1.6.2 Animal Tissue

- Before immersion in RNAlater® solution, cut large tissue samples to ≤0.5 cm in any single dimension. Small organs such as mouse liver, kidney and spleen can be stored whole in RNAlater® solution.
- Place the fresh tissue in 5–10 volumes of RNAlater® solution.
- Do not freeze samples in RNAlater® solution immediately; store at 4°C overnight (to allow the solution to thoroughly penetrate the tissue), remove supernatant, then move to –20°C or –80°C for long-term storage.

5.1.1.6.3 Tissue Culture Cells

- Pellet cells according to the protocols followed by your laboratory.
- Wash pellet once with cold PBS
- Remove supernatant and then add 5–10 volumes RNAlater® solution.
- Resuspend gently by using a p1000 pipette
- Store at –20°C or –80°C

5.1.1.6.4 Storage recommendations

- **Storage at –80°C**

  Samples in RNAlater® solution can be stored at –80°C indefinitely and is recommended. RNAlater® solution will freeze at –80°C. First incubate the samples in RNAlater® solution overnight at 4°C to allow thorough penetration of the tissue, then transfer to –80°C.
- **Storage at –20°C**

  Samples can be stored at –20°C indefinitely. Samples will not freeze at –20°C, but crystals may form; this will not affect subsequent RNA isolation. Incubate the samples in RNAlater® solution overnight at 4°C to allow thorough penetration of the

tissue, then transfer to –20°C. Samples can subsequently be thawed at room temperature and refrozen without affecting the amount or the integrity of the recoverable RNA.

- **Storage at 4°C**

  Most samples can be stored in RNAlater® solution at 4°C for up to 1 month without significant RNA degradation.

5.1.1.6.5 Send samples according to instructions in protocol NANOMMUNE SOP 5.2

5.1.1.6.6 Retrieve tissue from RNAlater® solution

5.1.1.6.6.1 Removal of RNAlater® solution from tissue

- Retrieve tissue from RNAlater® solution with sterile forceps,
- Quickly blot away excess RNAlater® solution with an absorbent lab wipe or paper towel
- Submerge the sample in RNA isolation lysis solution.
- Homogenize tissue promptly after placing it in lysis/denaturation solution.

5.1.1.6.6.2 Removal of RNAlater® solution prior to extraction RNA from cells

- Centrifuge samples at 5000 x g
- Gently take off supernatant
- Resuspend cells in appropriate buffer for RNA extraction

5.1.1.7 Reference

(1) RNAlater® manufacturer's website and instructions
http://www.ambion.com/techlib/prot/bp_7020.pdf

5.1.2 SOP NANOMMUNE 5.2v1 Protocol for sending microarray samples

5.1.2.1 Purpose

The preparation of samples for RNA microarray analysis by the Turku Center for Biotechnology.

5.1.2.2 Scope

This protocol is applicable to all members of the NANOMMUNE project and provides a descriptive procedure detailing the preparation, sending and ordering of microarray gene expression analysis on extracted RNA from cultured cells or tissue prepared by protocol SOP NANOMMUNE 5.1.

5.1.2.3 Principle

The prepared and shipped RNA samples are analyzed by Illumina BeadChips arrays (www.illumina.com), which allows large scale gene expression analysis for differential analysis, disease classification and pathway analysis. Isolated RNA extracted from cultured cells or tissue from different samples are hybridized on a single chip containing oligonucleotide bearing 3-micron beads in microwells etched into the surface of a slide-sized silicon substrate. Hybridization of biotin labelled cRNA to a probe sequence is used to identify the location of each bead. Subsequently the hybridized chips are scanned and analyzed by gene expression analysis software.

5.1.2.4 Reagents and Materials

1 µg RNA prepared by SOP 5.1 in sealed plastic tube.
Styrofoam transportation box.
5 kg carbon dioxide (dry ice)

5.1.2.5 Safety procedures and precautions

- Standard safety operating procedures are to be followed at all times.
- Treat all human-derived material as infectious.
- Lab coats and latex/vinyl/nitril gloves must be worn at all times.
- Dry ice is solidified carbon dioxide (CO_2) and is -79°C and should only be handled in well ventilated laboratories. Avoid contact with skin and eyes wear appropriate insulation gloves.

5.1.2.6 Procedure

- Perform RNA isolation according to protocol SOP NANOMMUNE 5.1
- Aliquot an equivalent of a minimum of 1 µg RNA.
- Freeze the samples in RNAlater® solution before sending!
- Delivery of the samples

- Pack samples in a closed and parafilm sealed eppendorf tube in a styrofoam box together with 5 kilograms of dry ice.
- Order a courier service and ensure that the package is delivered to Turku in 2-3 days to the following address:

  Turku Centre for Biotechnology/ Päivi Junni
  Tykistökatu 6 A
  FIN-20520 Turku

- Send the attachment A as print with the samples and electronically to paivi.junni@btk.fi and helena.ahlfors@btk.fi.
- Send the shipment tracking code to paivi.junni@btk.fi and helena.ahlfors@btk.fi.
- Take an action if the package won't be delivered in 3 days.
- If you have any questions about the sample preparation or sending the samples please contact us; paivi.junni@btk.fi or helena.ahlfors@btk.fi.

Attachments:

Attachment A: Project_information_form_NANOMMUNE

Attachment B1: Illumina arrays data sheets

Attachment B1:

Sentrix Human WG-6 Expression BeadChip (> 46,000 transcript-specific sequences)

Sentrix Human Ref-8 Expression BeadChip (> 23,000 RefSeq-based probe sequences/array)

Attachment B2:

Sentrix Human HT-12 Expression BeadChip (> 46,000 RefSeq-based probe sequences/array)

Attachment B3:

Sentrix Mouse WG-6 Expression BeadChip (> 47,000 transcript-specific probe sequences/array)

Sentrix MouseRef-8 Expression BeadChip (~24,000 RefSeq-based probe sequences/array