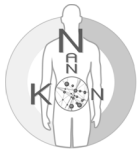
Responsible partner for this SOP: NanoKon - StauMa	Project Logo: 

Title: Profiling of the nanomaterial-protein corona

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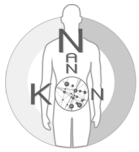
Responsible partner for this SOP: NanoKon - StauMa	Project Logo: 

Title: Profiling of the nanomaterial-protein corona

TABLE OF CONTENT

Title: Profiling of the nanomaterial-protein corona

1. PURPOSE

Quantitative and qualitative determination of the composition of the nanomaterial-protein corona following incubation in a (biological) fluid (e.g. human plasma).

2. OBJECTIVE

Due to their high free surface energy, most likely all nanomaterials adsorb (bio)molecules upon contact with any (biological) fluid. In particular, proteins rapidly bind the surface of nanoparticles forming a biological coating around the nanoparticle known as the protein corona. Hence, “nacked” nanomaterials in general are expected to exist in (biological) environments only for a short time (< 1min). Therefore, the biomolecule-coated nanomaterials need to be considered as novel materials with different properties compared to the pristine nanomaterials during their manufacturing. Particularly, the protein corona interacts with biological systems and thus, constitutes a major element of the biological identity of the nanoparticle. As such, the protein corona will also (co)determine nanotoxicology, including ecotoxicology, and may influence success or failure of biomedical/biotechnological applications.

Consequently, for the development and risk assessment of nanomaterials for any kind of application, it is therefore essential to analyze the protein corona, which is described in this SOP.

3. REGULATORY BASIS, REFERENCE DOCUMENTS

This assay protocol follows and is based on the information given in the following documents, but it is not strictly in accordance with each document in every point:

1. Tenzer, S., Docter, D., Kuharev, J., Musyanovych, A., Fetz, V., Hecht, R., Schlenk, F., Fischer, D., Kiouptsi, K., Reinhardt, C., Landfester, K., Schild, H., Maskos, M., Knauer, S.K., Stauber, R.H., 2013. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat Nanotechnol DOI: 10.1038.

3. Tenzer, S.; Docter, D.; Rosfa, S.; Wlodarski, A.; Kuharev, J.; Rekik, A.; Knauer, S. K.; Bantz, C.; Nawroth, T.; Bier, C.; Sirirattanapan, J.; Mann, W.; Treuel, L.; Zellner, R.; Maskos, M.; Schild, H.; Stauber, R. H., Nanoparticle size is a critical physicochemical determinant of the human blood plasma corona: a comprehensive quantitative proteomic analysis. ACS Nano 2011, 5, (9), 7155-6

4. Aggarwal, P.; Hall, J. B.; McLeland, C. B.; Dobrovolskaia, M. A.; McNeil, S. E., Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy. Adv Drug Deliv Rev 2009, 61, (6), 428-37.

5. Monopoli, M. P.; Aberg, C.; Salvati, A.; Dawson, K. A., Biomolecular coronas provide the biological identity of nanosized materials. Nat Nanotechnol 2012, 7, (12), 779-86.

6. Monopoli, M. P.; Walczyk, D.; Campbell, A.; Elia, G.; Lynch, I.; Bombelli, F. B.; Dawson, K. A., Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles. J Am Chem Soc 2011, 133, (8), 2525-34.

7. Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M., Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 2009, 8, (7), 543-57.

4. RELATED DOCUMENTS

Table 1 : References to documents needed to proceed according to this procedure

Document ID	Document Title

5 DEFINITIONS

Table 2: Glossary of Terminology used in the SOP

Term	Description
EDTA	Ethylenediaminetetraacetic acid
NP	Nanoparticle
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
IEF	Isoelectric focussing
DTT	Dithiothreitol
LC-MS	Liquid chromatography–mass spectrometry
Q-TOF	Quadrupole-time-of-flight
PLGS	ProteinLynx Global Server

6 PROCEDURE

a) Short description

NPs are incubated in blood plasma, centrifuged and washed. Bound proteins are then eluted from the NPs and the distinct protein binding profile is analyzed by 1D/2D-SDS-PAGE or LC-MS.

b) Materials and devices

- Blood plasma
- Sterile nanoparticle suspension
- Coomassie brilliant blue R-250 or related product
- ProteoExtract Kit (Merck, Darmstadt, Germany) or related product
- RapiGest (Waters, Eschborn, Germany) or related product
- Waters NanoAcquity UPLC System or related product
- Waters Q-TOF Premier API or related product
- Centrifuge (max. 13000xg)

c) Sample preparation

I. Preparation of NP-protein-samples using human plasma

a) Blood plasma collection:

Blood samples are taken from healthy donors. To prevent blood clotting use k_2-EDTA-coated tubes.

- Centrifuge the tubes for 5min at 4000 rpm to pellet red and white blood cells.
- The plasma supernatant can be pooled, aliquoted and stored at -80°C.

b) NP incubation:

The ratio of total particle-surface area to plasma concentration should be kept the same for the experiment to ensure comparability between the results. Calculate the total surface area ($A = 4ðR^2$) per particle mass for each of the individual NP to obtain a constant plasma volume to particle surface ratio.

- Incubate the particle suspensions with equal amount of human plasma for 1h at 4°C (total volume 500 µl).
- Centrifuge the samples to pellet the particle–protein complexes (10 min at 12000 rpm at 4°C).
- Resuspend the pellet in PBS, transfer to a new vial, and centrifuge again to pellet the particle-protein complexes. Repeat this procedure three times.
- Elute the proteins from the particles by adding SDS-sample buffer (62.5mM Tris-HCl pH 6.8; 2% w/v SDS, 10% glycerol, 50mM DTT, 0.01% w/v bromophenol blue) or lysis buffer for LC-MS (8 M Urea, 2% w/v CHAPS, 0.01% w/v bromophenol blue)

d) Detailed description of the procedure

II. SDS-PAGE

a) 1D-SDS-PAGE:

Discontinuous SDS-PAGE is carried out according to standard procedures (Tenzer 2011). Visualize the bound proteins by staining with Coomassie brilliant blue R-250.

b) 2D-SDS-PAGE:

Perform isoelectric focussing (IEF) and 2D-PAGE :

- Use 500 µg total protein for the IEF on the immobilized pH-gradient strips.
- Equilibrate the strips for 15min using equilibration buffer (50mM Tris, 6M Urea, 2% SDS, 30% Glycerol, 0.003% BPB; pH 8.8) including 1% DTT, following by 15 min incubation in equilibration buffer including 2.5% iodoacetamide.
- For the second dimension use a 14.5% SDS gel. To stain the gels use staining solution containing 2% HOAc, 0,001% SDS and 1/4000 Vol. SYPRO orange.
- To visualize protein spots use a fluorescence scanner at a PMT voltage of 1000.

III. Preparation of protein digests

- Precipitate proteins eluted from NPs (30µg) or crude plasma proteins (30µg) by using the ProteoExtract Kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions.
- Solubilize precipitated proteins in 25mM ammonium bicarbonate containing 0.1% RapiGest (Waters, Eschborn, Germany) for 15 min at 80°C.
- Reduce the proteins by adding 5mM DTT for 45 min at 56°C and free cysteines alkylated with iodoacetamide (Sigma, Taufkirchen, Germany) (15mM) for 1h at 25°C in the dark.
- Add 0.2µg porcine sequencing grade trypsin (Promega, Mannheim, Germany) and incubate the samples overnight at 37°C.
- Hydrolyze the RapiGest by adding 10mM HCl for 10 min at 37°C.
- Centrifuge the samples to remove the resulting precipitate at 13.000xg for 15min at 4°C.
- Transfer the supernatant into an autosampler vial for peptide analysis via LC-MS.

IV. LC-MS analysis of tryptic digests

Perform the capillary liquid chromatography of tryptic peptides with a Waters NanoAcquity UPLC system equipped with a 75µm x 150mm BEH C18 reversed phase column and a 2.6µL PEEKSIL-sample loop (SGE, Darmstadt, Germany). The aqueous mobile phase (mobile phase A) is H_2O (LC-MS Grade, Roth, Freiburg, Germany) with 0.1% formic acid. The organic mobile phase (mobile phase B) is 0.1% formic acid in acetonitrile (LC-MS grade, Roth).

- Load samples (2.6µL injection) onto the column in direct injection mode with 3% mobile phase B for 15 min at 400 nL/min, following by an additional 10min wash (3% B) for 10 min at 300 nL/min.
- Elute peptides from the column with a gradient from 3-35% mobile phase B over 90 min at 300nL/min following by a 20 min rinse of 80% mobile phase B.
- Re-equilibrate the column immediately at initial conditions (3% mobile phase B) for 20 min.
- Use [Glu1]fibrinopeptide as lockmass at 300fmol/µL.

To perform mass spectrometry analysis of tryptic peptides use a Waters Q-TOF Premier API system operated in V-mode with typical resolving power of at least 10,000.

- Sample the lock mass channel every 30s.
- Calibrate the mass spectrometer with a [Glu1]fibrinopeptide solution (300fmol/µL) delivered through the reference sprayer of the NanoLockSpray source.
- Collect accurate mass LC-MS data in an alternating, low energy (MS) and elevated energy (MSE) mode of acquisition.

e) Controls

- Blood plasma without NPs
- NPs without blood plasma incubation (neg. Ctrl for 1D-SDS-PAGE)

f) Data analysis

V. Bioinformatics, database searches and pathway analysis

For the processing of the continuum LC-MSE data and the database searches use the IDENTITYE- Algorithm of ProteinLynx Global Server (PLGS) version 2.4. Evaluate the resulting peptide and protein identifications by the software using statistical models as described (Tenzer 2011). To perform pathway analysis use GeneSpringGX11.0.2 (Waldbronn, Germany), IPA (Ingenuity System, Inc.; Redwood City, USA) as described (Tenzer 2011/2013).

g) Testing errors

7 SCOPE/AREA OF APPLICATION

Nano-toxicology, nano-ecotoxicology, nano-biomedicine/-biotechnology

For the development and risk assessment of nanomaterials for any kind of application, it is therefore essential to analyze the protein corona, which is described in this SOP.

8 ATTACHMENTS

9 HEALTH, SAFETY AND ENVIRONMENTAL CONSIDERATIONS

Follow standard safety operating procedures and local lab rules. Wear appropriate protective equipment, gloves, protective clothing and eyewear. Collect and dispose all waste in accordance with local laws.