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Identification of serum proteins bound to industrial nanomaterials

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ABSTRACT

Nanoparticles (NPs) are decorated with proteins and other biomolecules when they get into contact with biological systems. The presence of proteins in cell culture medium can therefore have effects on the biological outcome in cell-based tests. In this study, the manufactured nanomaterials silicon dioxide (SiO_2), titanium dioxide (TiO_2), iron-III-oxide (Fe_2O_3), and carbon black (CB) were used to study their interaction with single proteins from bovine and human plasma (albumin, fibrinogen and IgG) as well as with complete human serum. The protein binding capacity of the material was investigated and 1D gel electrophoresis was used to separate the bound proteins and to identify the bands by matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectrometry.

We found that the NP surface chemistry had a great impact on the amount of bound protein with distinct ligands for each of the tested particles. The hydrophobic CB NPs bound much more protein than the hydrophilic metal oxide NPs. Among the single proteins investigated, fibrinogen showed the strongest affinity for SiO_2, TiO_2 and CB NPs. The identified proteins from human serum adsorbed to these NPs were very different. Only apolipoprotein A1 was found to be adsorbed to all NPs.

These studies will help to explain the different degree of biological responses observed after *in vitro* exposure of cells in the absence or presence of serum and might also support the interpretation of *in vivo* experiments were NPs come directly into contact with blood plasma.

1. Introduction

Manufactured nanomaterials are decorated with proteins when they get into contact with biological systems (Nel et al., 2009). The term "protein corona "was coined to describe binding of plasma proteins to the surface of copolymer nanoparticles (NPs) (Cedervall et al., 2007; Lundqvist et al., 2008). The presence of proteins in cell culture medium containing serum or in plasma can affect the biological outcome of *in vitro* and *in vivo* experiments. For example, opsonization of polystyrene (PS) NPs correlates with increased uptake in the liver presumably by macrophages (Nagayama et al., 2007). Antibodies directed against the serum proteins complement C3 and immunoglobulin G could prevent uptake of PS NPs by macrophages *in vitro*. Also *in vitro* the presence of serum protects against the moderate toxicity induced by PS NPs in macrophages (Clift et al., 2010).

Less studied are the interactions of proteins with inorganic NPs as reviewed by Landsiedel et al. (2010). For gold NPs an evolution of the protein corona from rather loosely attached proteins towards an irreversible coating including serum albumin was recently demonstrated (Casals et al., 2010). The composition and thickness of the protein coating is also dependent on the cell culture medium as shown in the two media Roswell Park Memorial Institute medium (RPMI) and Dulbecco Modified Eagle's medium (DMEM). In RPMI medium the protein layer seems to be reduced and coincides with increased uptake and toxicity of gold NPs in the human cell lines HeLa and U937 (Maiorano et al., 2010). Unfortunately, for the environmentally more relevant metal and metal oxide NPs which are mainly derived from flame synthesis little is known with respect to protein interactions and the dependence on NP size and chemical composition (Landsiedel et al., 2010).

In this study, the manufactured nanomaterials silicon dioxide (SiO_2), titanium dioxide (TiO_2), iron-III-oxide (Fe_2O_3), and carbon black (CB) were used to study their interaction with single bovine and human blood proteins (albumin, fibrinogen and IgG) as well as with complete human serum dissolved in cell culture medium. Proteins adsorbed to the nanomaterials were separated from the unbound proteins by centrifugation. The protein content in the supernatant in relation to a sample without NPs indicates

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Table 1
Properties of the nanomaterials used in this study. Data are based on information from the manufacturers or suppliers if not stated otherwise. All nanomaterials are prepared by flame synthesis.

Chemical composition[a], primary size	Trade name, company	Crystalline phase	Surface area (BET m^2/g)	Surface chemistry, charge[c]	Shape, aggregation/ agglomeration[d]
SiO_2, 12 nm	Aerosil®200, Evonik	Amorphous	200 ± 25	Hydrophilic, negative	Spherical, yes
SiO_2, 40 nm	Aerosil®Ox50, Evonik	Amorphous	50 ± 15	Hydrophilic, negative	Spherical, yes
TiO_2, 5 nm	Alfa Aesar	Anatase	200–220	Hydrophilic, negative	Spherical, yes
TiO_2, 10 nm	Alfa Aesar	Anatase	100–130	Hydrophilic, negative	Spherical, yes
TiO_2, 32 nm	Alfa Aesar	Anatase	45	Hydrophilic, negative	Spherical, yes
TiO_2, 21 nm	P25, Evonik	Anatase/rutile	50 ± 15	Hydrophilic, negative	Spherical, yes
Fe_2O_3, 20–40 nm	NanoArc®, Alfa Aesar	Hematite	30–60	Hydrophilic, neutral	Spherical, yes
CB (carbon black), 14 nm [b]	Printex®90, Evonik	Amorphous	300	Hydrophobic, negative	Spherical, yes

[a] Purity was > 98%.
[b] Contains about 1% volatile fraction at 950 °C.
[c] Refs. Fubini et al. (2010) and Hussain et al. (2010).
[d] According to TEM analyses (see Fig. S1).

the depletion capacity of the material. The pellet was washed and bound proteins were further separated by 1D gel electrophoresis and identified using MALDI-TOF mass spectrometry after tryptic in-gel digest and Mascot database search.

2. Materials and methods

2.1. Material

Phosphate buffered saline without $Ca^{2+}$and Mg^{2+} (PBS) and RPMI 1640 medium without phenol red were purchased from Invitrogen (Karlsruhe, Germany). Bovine serum albumin (BSA) was from PAA laboratories (Cölbe, Germany) and human serum albumin (HSA), bovine and human fibrinogen (bFib, hFib), bovine immunoglobulin G (bIgG), Coomassie brilliant blue G-250 and other standard laboratory chemicals were from Sigma–Aldrich (Taufkirchen, Germany) unless otherwise stated. Human serum was bought from Lonza (Basel, Switzerland). The BCA protein assay was supplied by Thermo Fisher Scientific (Bonn, Germany), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) chemicals from Carl Roth (Karlsruhe, Germany) and trypsin (porcine) from Promega (Mannheim, Germany). Chemicals for MALDI-TOF MS were obtained from Sigma (Taufkirchen, Germany).

2.2. Particles

The nanomaterials used in this study, the suppliers and the data given by the suppliers are listed in Table 1. Further analysis of shape and agglomeration of NPs was performed by transmission electron microscopy (TEM) as described recently (Al-Rawi et al., 2011). The scheme of the procedure of the protein binding studies is shown in Fig. 1.

Particle stock solutions were freshly prepared at 10 mg/ml in RPMI 1640 medium without phenol red, vortexed and probe sonified for 30 s, duty cycle 50 (Branson Sonifier 250, G. Heinemann, Schwäbisch-Gmünd, Germany). This dispersion protocol was established in the lab for *in vitro* exposure of cell lines to nanoparticles (Fritsch-Decker et al., 2011). The protein solutions of 2 mg/ml and 20% (v/v) serum were mixed 1:1 (v/v) with the particle solutions in an Eppendorf vial yielding end concentrations of 5 mg/ml particles and 1 mg/ml protein or 10% serum, respectively. As a negative control, RPMI 1640 medium without particles was mixed with the protein solutions and treated as the particle dispersions to exclude effects due to adsorption to the vial or the centrifugation process.

The mixtures were vortexed and incubated at 37 °C for 1 h under constant shaking at 550 rpm. Not adsorbed proteins were separated by centrifugation at 21,000 × *g* for CB-NP. The centrifugation forces had to be lowered for mixtures containing SiO_2- and TiO_2-NP (2700 × *g*) and Fe_2O_3-NP (350 × *g*), otherwise the pellets could not be resuspended. The depletion of the protein concentration by particles was determined by measuring the protein concentration in an aliquot of the supernatant with the BCA assay and BSA as a standard.

Because washing with RPMI medium removed most of the adsorbed proteins in particular in case of Fe_2O_3 nanoparticles the pellets were washed three times with distilled water at the same volume as used for the incubation. Before the last centrifugation step, the particle suspensions were transferred into new vials. This prevented that proteins bound to the nanoparticles were mixed with proteins bound to the inner vial surface. For gel electrophoresis the NP-bound proteins were desorbed as indicated with reducing or non-reducing Laemmli solution, sonication and heating for 5 min at 95 °C.

2.3. Gel electrophoresis

The proteins in the solutions and in the particle pellets were dissolved in reducing or non-reducing Laemmli buffer, sonified in an ultrasonic bath, heated at 95 °C for 5 min and centrifuged for 5 min at 2700 × *g*. Aliquots of the supernatants as indicated in the figures were loaded on 6% or 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). After electrophoresis, the gels were stained in Coomassie staining solution [0.25% (w/v) Coomassie Brilliant Blue G-250 in methanol:acetic acid:water at 9:2:9 (v:v:v)] for 1 h at room temperature and destained in methanol:acetic acid:water at 9:2:9 (v:v:v). The gels were scanned with an Odyssey® Infrared Imaging System at 700 nm and the protein bands were quantified with the Odyssey® software (LICOR Biosciences, Bad Homburg, Germany). The protein amount of a band was estimated from its optical density in comparison to the optical density of a BSA band of known protein amount.

2.4. In-gel digestion of proteins

Coomassie-stained bands were excised from the polyacrylamide gel and washed with 10 mM ammonium hydrogen carbonate (solution 1) and destained by incubation in solution 2 [1:1 (v/v) solution of acetonitrile with 10 mM ammonium bicarbonate] for 10 min each under constant shaking at 450 rpm. Disulfide bond cleavage was performed with 5 mM dithiothreitol in 10 mM ammonium hydrogen carbonate at 60 °C for 15 min. Incubation with 55 mM iodine acetamide at room temperature for 15 min was performed to alkylate the SH-groups. The gel pieces were washed with wash solutions 1 and 2 for 10 min each. This was repeated three times followed by drying the gel pieces in a SpeedVac at 45 °C for 20 min. The dryed gel pieces were soaked in 2–10 μl of 25 ng/μl trypsin solution (1:3 mixture (v/v) of 100 ng/l trypsin and 10 mM ammonium hydrogen carbonate). The volume of the trypsin solution depends on the size of the gel pieces. After uptake of the solution the gel pieces were covered with 10–30 μl of 10 mM ammonium bicarbonate and incubated overnight under constant shaking at 450 rpm. On the following day, the samples were sonified in an ultrasonic bath for 10 min. 0.5 μl of a sonicated sample was mixed with 0.5 μl of matrix, spotted on a stainless steel MALDI carrier tray and analysed after crystallinisation. In case of very weakly stained protein bands the digested samples were concentrated by Zip-Tip pipette tips containing C18 reversed-phase (Millipore, Schwalbach, Germany).

2.5. MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed on a 4800 MALDI-TOF/TOF instrument from Applied Biosystems (Framingham, MA). Peak lists were generated using Data explorer software 4.0 (Applied Biosystems) and calibrated against a peptide standard mixture (des-Arg-Bradykinin, m/z = 904.4681; Angiotensin I, m/z = 1296.6853; Glu-Fibrinopeptide B, m/z = 1570.6774; ACTH clip 1–17, m/z = 2093.0867; and ACTH clip 18–39, m/z = 2465.1989). The tryptically digested proteins were identified using MASCOT search engine (http://www.matrixscience.com) with NCBI (National Center for Biotechnology Information), MSDB and Swissprot database with a mass tolerance < 0.1 Da for the