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Short Communication

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Uptake of nanoparticles by alveolar macrophages is triggered by surfactant protein A

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Abstract

Understanding the bio-nano interactions in the lungs upon the inhalation of nanoparticles is a major challenge in both pulmonary nanomedicine and nanotoxicology. To investigate the effect of pulmonary surfactant protein A (SP-A) on the interaction between nanoparticles and alveolar macrophages, we used magnetite nanoparticles (110–180 nm in diameter) coated with different polymers (starch, carboxymethyldextran, chitosan, poly-maleic-oleic acid, phosphatidylcholine). Cellular binding and uptake of nanoparticles by alveolar macrophages was increased for nanoparticles treated with SP-A, whereas albumin, the prevailing protein in plasma, led to a significant decrease. A significantly different adsorption pattern of SP-A, compared to albumin was found for these five different nanomaterials. This study provides evidence that after inhalation of nanoparticles, a different protein coating and thus different biological behavior may result compared to direct administration to the bloodstream.

From the Clinical Editor: In this nano-toxicology study of inhaled nanoparticles, the authors investigated the effect of pulmonary surfactant protein A on the interaction between nanoparticles and alveolar macrophages utilizing magnetite nanoparticles coated with different polymers (starch, carboxymethyldextran, chitosan, poly-maleic-oleic acid, phosphatidylcholine). Cellular binding and uptake of nanoparticles increased for nanoparticles treated with SP-A, whereas albumin, the prevailing protein in plasma, led to a significant decrease.

Key words: SP-A; Protein adsorption; Macrophage clearance

Interactions of nanomaterials with complex biological molecules are most definitely a high-priority topic to ensure safety and applicability of nanotechnology,[1] and are therefore most important in both nanomedicine and nanotoxicology. The lungs especially, with a large surface area (140 m^2), are considered to be the organ with highest relevance in terms of nanoparticle exposure,[2] and also serve as an interesting route for drug delivery.[3]

Various studies in the past have addressed nanoparticle inhalation and demonstrated the possibility of deposition in the peripheral lungs. However, there is still a lot to be understood about the actual intermediate steps between deposition and biodistribution; that is, what happens after landing of nanoparticles in the respiratory region, and how do they interact with the air-blood barrier? The first biological surface encountered by nanoparticles deposited on the alveolar epithelium is pulmonary surfactant, a complex mixture constituted by 90% lipids, mainly phospholipids, and 5–10% proteins. Among the protein moieties, four pulmonary surfactant-associated proteins (SPs) are known: SP-A, -B, -C, and -D.[4]

Especially the most prevalent protein, SP-A (650 kD), which is able to bind a variety of biological patterns,[5] is likely to play a key role in adsorption to nanoparticles and related biological effects. Acting as a broad-spectrum opsonin, SP-A can greatly influence the activity of alveolar macrophages (AMs) and other cells in the alveolar tissue.[5] Protein adsorption to nanoparticles

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Table 1
Size and zeta potential of different chemically modified magnetic nanoparticles (mNP) in relevant media

Surface modification	Peak mean of volume-based size distribution (nm)*			Zeta potential (mV)‡		
	in Milli-Q water	+ BSA†	+ SP-A†	in Milli-Q water	+BSA§	+SP-A§
Starch	138.7 ± 1.8	139.5 ± 2.0	135.5 ± 0.8	−8.8 ± 0.6	−3.1 ± 0.3	−3.7 ± 0.4
Carboxymethyldextran	183.2 ± 1.0	171.4 ± 2.1	170.5 ± 1.1	−35.0 ± 1.3	−27.3 ± 1.3	−29.9 ± 1.6
Chitosan	164.4 ± 1.2	696 ± 159	856 ± 188	42.4 ± 1.1	−17.6 ± 0.1	−22.8 ± 1.3
Poly-maleic-oleic acid	114.8 ± 1.3	1057 ± 159	1063 ± 412	29.8 ± 2.4	−8.6 ± 0.6	−15.4 ± 0.2
Phosphatidylcholine	130.6 ± 2.1	145.4 ± 7.7	135.2 ± 5.1	−44.9 ± 3.6	−31.8 ± 0.6	−39.2 ± 1.8

* Peak means of volume-based size distributions were determined using a Zetasizer Nano-ZS (Malvern Instruments) and are displayed as mean ± SD (n = 3).
† For each measurement mNPs were incubated for 10 minutes at room temperature in Milli-Q water supplemented with either BSA (1 mg/mL) or RPMI + SP-A (10 μg/mL).
‡ Zeta potentials were determined using a Zetasizer Nano-ZS (Malvern Instruments) and are displayed as mean ± SD (n = 3).
§ Samples were measured after 5 minutes incubation at 25°C in 10-fold-diluted Tris-NaCl (pH 7.4) supplemented with either BSA or SP-A (0.002% w/v final protein concentration).

in the peripheral lungs is a crucial intermediate effect between nanoparticle deposition on the one hand, and particle clearance, translocation, or also toxic effects on the other hand. Astonishingly, however, the role of surfactant proteins in this context has only been marginally addressed so far.

In this study we used magnetic nanoparticles (mNPs) with different chemically modified surfaces (Table 1) to explore the resulting biological effects mediated by SP-A. We studied binding and uptake by AMs using flow cytometry analysis and confocal laser scanning microscopy (CLSM). For comparison, albumin (bovine serum albumin, BSA) was used as the prevailing protein in plasma. Furthermore, adsorption of SP-A and albumin to the mNPs was investigated.

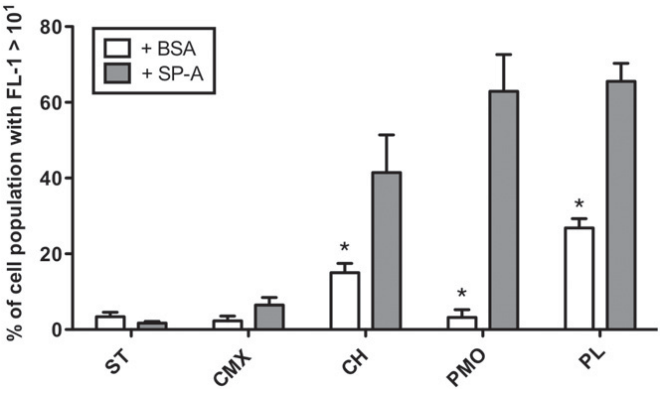

Figure 1. Influence of BSA and SP-A on association of mNPs (ST, starch; CMX, carboxymethyldextran; CH, chitosan; PMO, poly-maleic-oleic acid; PL, phosphatidylcholine; diameter 110–180 nm) with AMs studied by flow cytometry. Data represent mean ± SE from at least three experiments. *Indicates a significant difference compared to SP-A ($P < 0.05$).

Methods

Interaction of mNPs with AMs

Murine AMs (MH-S; 2×10^5 cells per well) were seeded in 24-well plates. All experiments were carried out in cell culture media free of fetal calf serum (RPMI w/o FCS) to discriminate the mediated effects of either protein separately. Before each experiment, MH-S were washed with phosphate buffered saline (PBS) and equilibrated for 30 minutes in RPMI w/o FCS at 37°C. Aqueous mNPs suspension was incubated for 20 minutes at 37°C in RPMI w/o FCS supplemented with either BSA or isolated SP-A. Pre-incubated mNP formulations were then added to MH-S cells, resulting in a final concentration of 1×10^{10} mNPs/mL, and incubated for 90 minutes at 37°C under gentle shaking. After incubation, cells were washed twice with PBS, and mNP–MH-S association was determined using flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, New Jersey).

Dynamic light scattering (DLS) and zeta potential

Hydrodynamic diameters of mNPs in MilliQ water (Millipore, Billerica, Massachusetts), supplemented with RPMI 1640 (RPMI) with BSA or SP-A, were determined by means of DLS, and zeta potentials of mNPs in MilliQ water, 10-fold-diluted Tris-NaCl buffer (5 mM Tris, 150 mM NaCl, pH 7.4) with BSA or SP-A, were measured by laser Doppler velocimetry using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, United Kingdom).

Protein-binding assay

To study the adsorption of BSA and SP-A, mNPs were incubated in protein solutions of BSA, or isolated SP-A, respectively, in Tris-NaCl buffer, resulting in a final mNP-to-protein ratio of approximately 2:1 (w/w). After 20 minutes incubation at 37°C, mNPs were separated from unbound proteins using a M2 magnet separator (Bilatec, Viernheim, Germany). Proteins of interest (bound proteins) were desorbed from mNPs under denaturing conditions. Samples were analyzed under reducing conditions on 12% sodium dodecyl sulfate–polyacrylamide gels and stained with colloidal Coomassie (Fermentas, St. Leon-Rot, Germany). Protein concentration was determined from band intensity and expressed as bound protein in percent of control.

Visualization and quantification of particle association and uptake

MH-S (2×10^5 cells per well) were seeded in 24-well imaging plates (zell-kontakt, Nörten-Hardenberg, Germany). Experiments

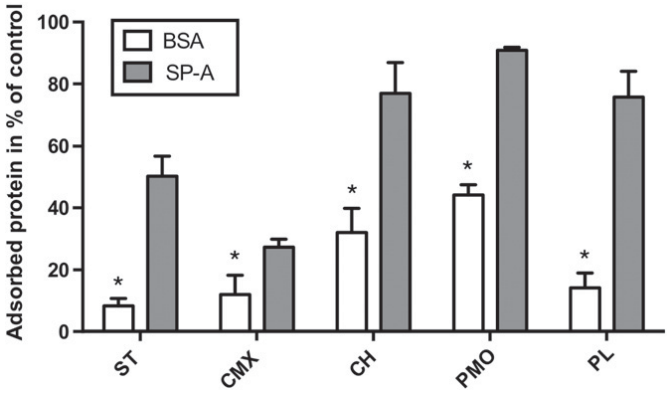

Figure 2. Protein adsorption to mNPs after 20 minutes' incubation in BSA or SP-A as determined by densitometry. Data represent mean of at least three independent experiments ± SE. *Indicates the level of significant difference compared to SP-A ($P < 0.05$).

were performed in analogy to flow cytometry–based assays. After 90 minutes incubation at 37°C under gentle shaking, cells were washed twice with PBS. Membranes were subsequently stained with Rhodamine *Ricinus communis* agglutinin I (Vector Laboratories, Burlingame, California). After fixation with formaldehyde, samples were visualized and analyzed in z-direction (z-stacks) using a Zeiss LSM 510 with META detector (Carl Zeiss AG, Jena, Germany).

For respective protocol details see Supplementary Material, available online at http://www.nanomedjournal.com.

Results

The effect of SP-A and BSA, respectively, on the cellular binding and uptake of mNPs was studied in an immortalized mouse AM model (Figure 1). In case of chitosan (CH)-, poly-maleic-oleic acid (PMO)-, and phosphatidylcholine (PL)-mNPs, a significantly increased cell association was observed for SP-A compared to BSA. In contrast, starch (ST)- and carboxymethyl-dextran (CMX)-mNPs showed no differences in cell association for the two proteins, and remained at rather low levels.

To clarify these findings, we studied the adsorption of SP-A and BSA to the mNPs. For all mNPs, adsorption of BSA was significantly lower than SP-A, and a material-dependent effect was demonstrated (Figure 2). Especially in case of PL-mNPs, the different binding of these two proteins was most pronounced, being almost five times higher for SP-A compared to BSA, which was on the same level as for the two BSA low-binding mNPs (ST- and CMX-mNPs).

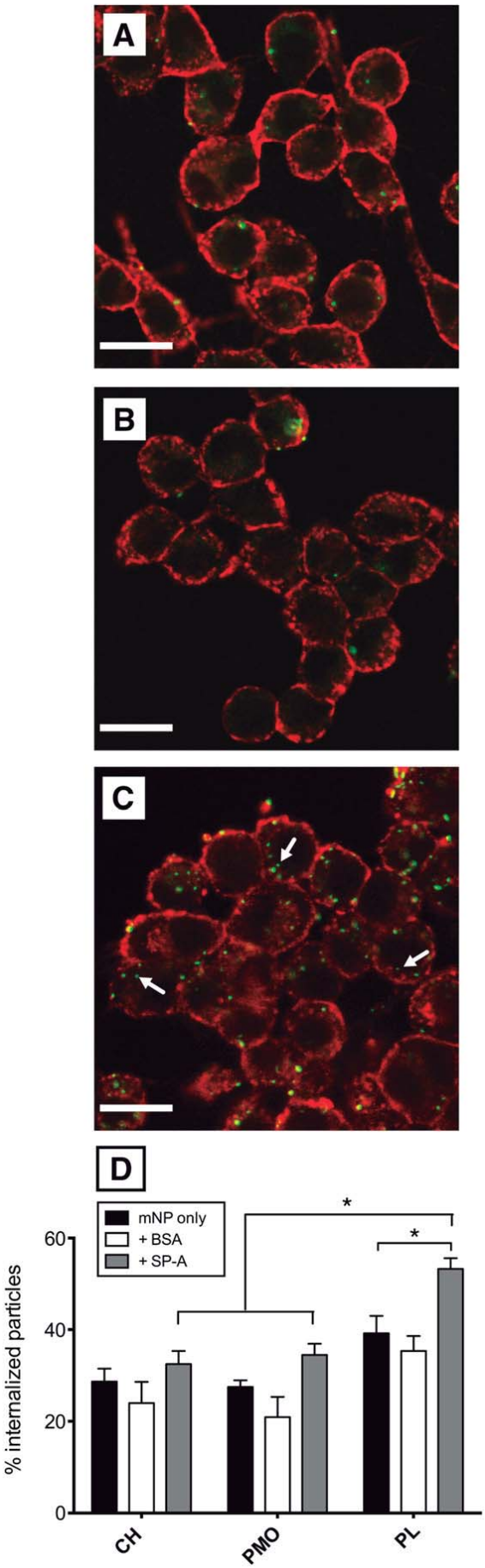

Figure 3. Effect of SP-A on mNPs uptake by AMs studied by CLSM. **(A–C)** Representative images of MH-S cells (membrane in red) exposed to PL-mNPs (mNPs in green) in the absence of protein **(A)** or in the presence of BSA **(B)** or SP-A **(C)**, respectively. **(D)** A quantitative image analysis using micrographs recorded in the equatorial plane of the cells was conducted to discriminate between internalized and cell-adherent mNPs. mNP number inside membrane boundary was counted (see white arrows for representative mNPs) and expressed as percent of total mNP number (compare Supplementary Material). Scale bars indicate a distance of 16 μm. Data represent mean ± SE from at least four experiments (BSA two experiments). *Indicates the level of significant difference ($P < 0.05$).

Furthermore, we chose CLSM to discriminate between internalized and cell-adherent mNPs (Figure 3). Here we decided to focus only on the most reactive CH-, PMO-, and PL-mNPs to study the effect exerted by SP-A versus BSA on the internalization by AMs. Quantitative image analysis revealed that pretreatment of mNPs with BSA tended to decrease internalization for each tested mNP in comparison to protein-free medium ("mNP only"), whereas SP-A increased it (Figure 3, *D*). The increased uptake under influence of SP-A was most pronounced for PL-mNPs, reaching statistical significance at $P < 0.05$. SP-A–induced cellular uptake was, moreover, most pronounced for PL-mNPs compared to CH- and PMO-mNPs ($P < 0.05$).

Additionally, agglomeration behavior of mNPs was studied in respective test media using DLS. Though ST- and CMX-mNPs maintained colloidal stability in all test media, larger agglomerates were observed for CH- and PMO-mNPs when dispersed in media containing either BSA or SP-A. In contrast, PL-mNPs, which had previously shown high adsorption of SP-A but low binding of BSA, remained colloidally dispersed in each test medium (Table 1).

Discussion

Our data clearly show that SP-A leads to a significant increase of mNP interaction with AMs (Figure 1). Such effects, however, were not observed for BSA. These findings were supported when the binding of these two proteins to the mNPs was studied. Here we could show that SP-A binding was higher for all nanoparticles tested, when compared to BSA. Furthermore, some mNPs showed lower SP-A binding than others, pointing out that the primary surface material of the NPs greatly influences the extent of protein adsorption (Figure 2). Such a material-dependent adsorption of SP-A was also found in a recent study from our group, investigating the binding of SP-A to different metal oxide nanomaterials.[6] The significantly higher binding of PMO- and PL-mNPs (i.e., the two mNPs with the highest SP-A binding) might be partly explained with this general preference of SP-A for lipophilic patterns.[7] Furthermore, the observation that PL-mNPs demonstrated high SP-A binding could be linked to the naturally high phospholipid association of SP-A in pulmonary surfactant.[8] By comparison of the in vitro data (AM interaction) and protein adsorption, we can speculate that SP-A forms a more effective protein coating with greater potential to trigger the mNPs interaction with AMs. BSA, however, does also adsorb to some extent to the mNPs, but seems to shield free surface groups of the mNPs, and prevents them thereby from binding to cellular membranes.

Besides the specific effects exerted by adsorbed proteins, it is well to consider the influence of protein binding on other particle characteristics, such as size or surface charge. Here our results reveal changes in zeta potential upon protein adsorption. The more negative values (Table 1), compared to those measured in BSA-containing media, are well explained by the adsorption of SP-A, in that this protein is negatively charged at physiological pH as a result of its isoelectric point of pH 4–5.[9] Also, the formation of particle agglomerates for some mNPs, namely CH- and PMO-mNPs, was observed (Table 1). Such an increase in particle size is likely to affect the mNP deposition onto the cells, thereby enhancing cell interactions.[10] Additionally, it is known that a size of 1–5 μm increases binding and uptake by AMs.[11] These findings emphasize that, especially in the case of CH- and PMO-mNPs, both particle size and SP-A might contribute to the high AM interaction. However, SP-A–binding PL-mNPs still remained dispersed as nanoparticles, regardless of the presence of any protein studied (Table 1). Especially for these nanoparticles, it is therefore very likely that the observed biological effects are mediated by adsorbed SP-A, because the cell association here was significantly higher than when preincubated with BSA (Figure 1). Furthermore, SP-A significantly increased the uptake of PL-mNPs compared to protein-free medium (Figure 2, *D*). These results lead us to the conclusion that nanoparticles with high binding to SP-A, though retaining their colloidal stability, seem to bear great potential to interact more specifically with AMs, favoring their internalization and clearance.

Overall, our results indicate that when nanoparticles make first contact with a protein, which is highly relevant for the peripheral lung (i.e., SP-A), they undergo a different protein priming than when they come in contact with a component of the bloodstream (BSA). Under the assumption that they may act as "second messengers," the proteins adsorbed to a nanoparticle in the primarily encountered physiological compartment—whether intended or accidentally—may be very decisive for the subsequent biodistribution and other biological effects.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nano.2011.07.009.

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