European Journal of Pharmaceutics and Biopharmaceutics 77 (2011) 376–383

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Interaction of metal oxide nanoparticles with lung surfactant protein A

Christine Schulze [a], Ulrich F. Schaefer [a], Christian A. Ruge [a], Wendel Wohlleben [b], Claus-Michael Lehr [a,c,*]

[a] Department of Biopharmaceutics and Pharmaceutical Technology, Saarland University, Saarbruecken, Germany
[b] BASF SE, Polymer Physics Research, GKP/O – G201, Ludwigshafen, Germany
[c] Department of Drug Delivery (DDEL) Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS) Helmholtz Center for Infection Research (HZI), Saarland University, Campus A 4 1, Saarbruecken, Germany

ARTICLE INFO

Article history:
Available online 5 November 2010

Keywords:
Surfactant protein A
Nanoparticles
Particle–protein interaction
Protein adsorption
Deagglomeration
BCA assay

ABSTRACT

The alveolar lining fluid (ALF) covering the respiratory epithelium of the deep lung is the first biological barrier encountered by nanoparticles after inhalation. We here report for the first time significant differences for metal oxide nanoparticles to the binding of surfactant protein A (SP-A), the predominant protein component of ALF. SP-A is a physiologically most relevant protein and provides important biological signals. Also, it is involved in the lung's immune defence, controlling e.g. particle binding, uptake or transcytosis by epithelial cells and macrophages. In our study, we could prove different particle–protein interaction for eight different nanoparticles, whereas particles of the same bulk material revealed different adsorption patterns. In contrast to other proteins as bovine serum albumin (BSA), SP-A does not seem to significantly deagglomerate large agglomerates of particles, indicating different adsorption mechanisms as in the well-investigated model protein BSA. These findings may have important consequences for biological fate and toxicological effects of inhaled nanomaterials.

1. Introduction

When nanoparticles come into contact with biological systems, their interactions with proteins is of utmost importance. As it is known that small particles are taken up in higher amounts than larger ones, the level of particle uptake into cells could be altered due to protein coating of particles. Dispersion of nanoparticles in protein solution can drastically alter the dispersion characteristics, leading to either increased or decreased particle sizes. We have previously demonstrated that the addition of fetal calf serum leads to deagglomeration [1] and hence to smaller particle sizes for some of the particles tested here. Also, it could be demonstrated by Ehrenberg and co-workers that particles coated with serum proteins adsorbed onto endothelial cells in higher amounts as uncoated ones [2]. Hence, the phenomenon of protein adsorption onto nanoparticles entering biological systems could lead to significant toxicological consequences and must be investigated thoroughly.

The Dawson group has pioneered structure–property relationships in protein coronas during the last few years using plasma proteins [3–6]. However, there is general consensus in nanotoxicology that inhalation represents the most relevant route of exposure [7]. In this case, the first biological barrier that inhaled particles will encounter is the pulmonary surfactant on top of the alveolar lining fluid (ALF), an ultra thin liquid layer covering the respiratory epithelium towards the air side. The pulmonary surfactant consists of approximately 90% lipids (mainly phospholipids) and 10% proteins (so-called surfactant proteins, SP) by weight [8]. Concerning the interaction between the pulmonary surfactant and inhaled particles, we assume that especially the four so-called lung surfactant proteins play a key role. Surfactant protein B and C are very lipophilic and improve the surface activity of surfactant phospholipids [9]. The more hydrophilic surfactant proteins A and D (SP-A, SP-D) belong to the collectins recognizing, binding and facilitating the clearance of infectious particles from the lung [10]. As SP-A is the most prominent of the four surfactant proteins and because of its major role in lung immune defence, a possible particle interaction with this protein is highly important to understand and to predict further biological responses. The adsorption of pulmonary surfactant components has already been addressed in several studies for diesel soot, quartz and kaolin [11] as well as for gold [12], TiO_2 and polystyrene nanoparticles [13,14] but they predominantly concentrated on the lipid fraction of pulmonary

Abbreviations: ALF, alveolar lining fluid; SP-A, surfactant protein A; BSA, bovine serum albumin; pBALF, porcine broncheoalveolar lavage fluid; BCA assay, bicinchinoninic acid protein quantification assay; AUC, analytical ultracentrifugation; CRD, carbohydrate recognition domains; SDS–PAGE, sodiumdodecylsulfate–polyacryl-gelelectrophoresis; SPs, surfactant proteins; FCS, fetal calf serum; CNTs, carbonanotubes.

* Corresponding author. Department of Biopharmaceutics and Pharmaceutical Technology, Saarland University, D-66041 Saarbruecken, Germany. Tel.: +49 681 302 2039; fax: +49 681 302 4677.

E-mail addresses: chr.schulze@mx.uni-saarland.de (C. Schulze), ufs@mx.uni-saarland.de (U.F. Schaefer), c.ruge@mx.uni-saarland.de (C.A. Ruge), wendel.wohlleben@basf.com (W. Wohlleben), lehr@mx.uni-saarland.de (C.-M. Lehr).

0939-6411/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2010.10.013

surfactant. Also, with the exception of TiO_2, quartz and kaolin, those particles are hardly transferable to materials that are handled at kiloton scale already, such as metal oxide nanoparticles. Therefore, we decided to study the interaction of metal oxide nanoparticles with lung surfactant protein A present in porcine broncheoalveolar lavage fluid (pBALF), as pBALF is a well known and widely used source for surfactant proteins.

To investigate nanoparticles–protein interactions, microcalorimetry and surface plasmon resonance technique were introduced by Cedervall et al. using co-polymer particles as model [15]. However, we found these techniques not readily transferable to industrially relevant nanomaterials, such as metal oxides, mainly due to rapid sedimentation. Hence, we adopted the colorimetric bicinchinoninic acid protein quantification assay (BCA assay), gel electrophoresis and immunoblotting to quantify and identify the interacting proteins.

2. Materials and methods

2.1. Determination of protein adsorption onto metal oxide nanoparticles via BCA assay

One hundred and forty-eight milligrams of nanoparticles was dispersed with 2 ml of a 1:10 diluted pBALF solution (leading to a protein concentration of 7.4 mg/ml; preparation see section 1b), leading to particle–protein ratio of 10:1. The resulting dispersions were stirred at room temperature for 1 h at 300 rpm, transferred into Eppendorf tubes and centrifuged at 23,000*g* for 45 min at 10 °C in a Hettich Universal 30 RF with rotor E1175. Afterwards, the supernatants were used for BCA assay as described in the manual (Bicinchinoninic Acid Kit for Protein Determination, Sigma, Cat. No. BCA1-1KT). The resulting protein concentrations in the supernatants were subtracted from the original concentration and related to the negative control (centrifuged protein solution without particles).

2.2. Preparation of porcine broncheoalveolar lavage fluid (pBALF)

pBALF preparation was modified after Taeusch et al. [55]. In short, three porcine lungs, derived from a local butcher and removed in toto, were each filled with about 0.6 l of cold (4 °C) purified water and gently massaged for about 5 min. We used water instead of buffer to avoid any influence of salts onto the adsorption process. Then, the fluid was removed and collected; the fluid of all lungs was pooled and centrifuged at 1400 rpm for 4 min to remove cellular residues. The thus obtained volume of about 2 l of pBALF was frozen at −80 °C until lyophilisation in a Christ Alpha 2-4 LSC lyophilisation device and rediluted in 200 ml of purified water in order to concentrate the proteins. The protein content was determined with BCA assay to be 74.03 mg/ml. The content of SP-A was proven by western blotting and immunostaining, performed as described later, in comparison with literature [17,49] and a SP-A reference. The pBALF was aliquoted and stored at −80 °C until use.

The time frame between picking up the lungs and preparation of the pBALF was about 45 min. During the whole transportation process, the lungs were cooled on ice to preserve the tissue. After they arrived in the laboratory, we started pBALF preparation immediately.

2.3. SDS–PAGE, western blotting and immunostaining

The contents and preparation of all buffers and solutions are summarized in Table 1. After incubation with the nanoparticles in a particle–protein relation of 10:1 and centrifugation as described for BCA assay, the supernatants were removed, the pellets rinsed three times with purified water and resuspended with 0.5 ml of purified water. A volume of 0.1 ml of the supernatants and pellet dispersions, respectively, was mixed with 0.1 ml of 2x sample buffer and denatured for 5 min at 95 °C to detach the proteins from the particles. Then, 20 μl of each sample was applied to a 12% polyacrylamide gel (4.5 ml of purified water, 2.5 ml of separating gel buffer, 3 ml of acrylamide solution (Rotiphorese Gel 40 (29:1), Carl Roth GmbH & Co, Cat. No. A515.1), 0.05 ml of ammoniumperoxodisulfate (APS; Carl Roth GmbH & Co, at. No. 95923), 0.005 ml of Temed (Carl Roth GmbH & Co, Cat. No. 23673)), covered with a 4% stacking gel (2.5 ml of purified water, 0.95 ml of stacking gel buffer, 0.4 ml of acrylamide solution, 0.0225 ml of APS, 0.0075 ml of Temed) and run, soaked in running buffer, for 110 min at 100 V in a BioRad MiniProtean II.

The stacking gels were removed and the separating gels covered with nitrocellulose membranes (Protran BA 85 Nitrocellulose, Whatman, Cat. No. 10401197), sandwiched in filter paper and soaked in blotting buffer. After removing air bubbles from the layer interspaces, the blotting sandwiches were transferred into a Bio-Rad Mini Trans-blot Cell and tank-blotted in blotting buffer at 300 mA for 90 min. As the protein marker (Spectra Multicolor, Broad Range Protein Ladder, Fermentas, Cat. No. SM1849) was prestained, there was no need to check the protein transfer by Ponceau staining.

The membranes were blocked for 2 h in blocking buffer and then they were incubated with rabbit anti-surfactant protein A at a dilution of 1:2500 in blocking buffer (Anti-Surfactant Protein A, Millipore, Cat. No. AB 3424) for 2 h under gentle luffing. The blots were washed three times with TBS buffer for 10 min prior to incubation with alkaline phosphatase–conjugated goat anti-rabbit IgG (Goat anti-Rabbit IgG, Alkaline Phosphatase Conjugated, Millipore, Cat. No. AP132A) and diluted 1:5000 in blocking buffer. After washing three times for 10 min with TBS buffer, the blots were developed in 10 ml of NBT-BCIP dying solution for several minutes. Finally, the blots were scanned and saved as .tiff files.

2.4. Agglomeration control by analytical ultracentrifugation (AUC)

The particle size distribution was determined by analytical ultracentrifugation (AUC) of ~500 μL of the test dispersion with a mass ratio of nanomaterial:BALF proteins = 2:1. This ratio corresponds to around 10 mg/cm^2 protein mass concentration per nanomaterial surface for the metal oxides, because these have all similar values of the BET surface. This ratio in the solution was chosen because this situation is close to a particulate contamination, in the sense that the reservoir of surfactant proteins is not depleted: Only a small part of the available protein mass has adsorbed.

Simultaneous detection by synchronized interference optics (Beckmann, model XLI) quantified the amount and the diameter of each fraction independently from 1 nm up to several microns diameter [7,56,57]. We can thus successively quantify in a single measurement the protein content, the protein molar mass, the nanomaterial content and the nanomaterial state of agglomeration, presented as double-logarithmic plot in Fig. 2. When the retrieved concentration of proteins is less than 100 wt% at the expected molar mass, we assume that the remaining proteins have adsorbed to a particulate surface. When the retrieved concentration of nanomaterial is less than 100 wt% in the measurement interval, we assume that the remainder has agglomerated. The evaluation of the AUC raw data incorporated the fractal morphology of nanoparticle agglomerates and applied the fractional dimension of 2.1 together with the sedimentation relation as specified in Eq. (6) of Ref. [58]. This value of the fractional dimension has been shown to be universal for all reaction-limited colloid agglomerates