European Journal of Pharmaceutics and Biopharmaceutics xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Flotillin-involved uptake of silica nanoparticles and responses of an alveolar-capillary barrier *in vitro*

Jennifer Kasper [a,*], Maria I Hermanns [b], Christoph Bantz [c], Stefanie Utech [c], Olga Koshkina [d], Michael Maskos [c,d,e], Christoph Brochhausen [a], Christine Pohl [a], Sabine Fuchs [f], Ronald E. Unger [a], C. James Kirkpatrick [a]

[a] *University Medical Centre, Institute of Pathology, Mainz, Germany*
[b] *ikfe GmbH, Institut für klinische Forschung und Endwicklung, Mainz, Germany*
[c] *Johannes Gutenberg University Mainz, Institute of Physical Chemistry, Mainz, Germany*
[d] *BAM, Federal Institute for Materials Research and Testing, Berlin, Germany*
[e] *Institut für Mikrotechnik, Mainz, Germany*
[f] *Experimental Trauma Surgery, University Medical Center Schleswig-Holstein, Kiel, Germany*

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Silica nanoparticles
Alveolar-capillary barrier
NP uptake
NP-transport
Endocytosis
Flotillin-1/-2-dependent uptake/trafficking

ABSTRACT

Drug and gene delivery via nanoparticles across biological barriers such as the alveolar-capillary barrier of the lung constitutes an interesting and increasingly relevant field in nanomedicine. Nevertheless, potential hazardous effects of nanoparticles (NPs) as well as their cellular and systemic fate should be thoroughly examined. Hence, this study was designed to evaluate the effects of amorphous silica NPs (Sicastar) and (poly)organosiloxane NPs (AmOrSil) on the viability and the inflammatory response as well as on the cellular uptake mechanisms and fate in cells of the alveolar barrier. For this purpose, the alveolar epithelial cell line (NCI H441) and microvascular endothelial cell line (ISO-HAS-1) were used in an experimental set up resembling the alveolar-capillary barrier of the lung. In terms of IL-8 and sICAM Sicastar resulted in harmful effects at higher concentrations (60 μg/ml) in conventional monocultures but not in the coculture, whereas AmOrSil showed no significant effects. Immunofluorescence counterstaining of endosomal structures in NP-incubated cells showed no evidence for a clathrin- or caveolae-mediated uptake mechanism. However, NPs were enclosed in flotillin-1 and -2 marked vesicles in both cell types. Flotillins appear to play a role in cellular uptake or trafficking mechanisms of NPs and are discussed as indicators for clathrin- or caveolae-independent uptake mechanisms. In addition, we examined the transport of NPs across this *in vitro* model of the alveolar-capillary barrier forming a tight barrier with a transepithelial electrical resistance of 560 ± 8 Ω cm^2. H441 in coculture with endothelial cells took up much less NPs compared to monocultures. Moreover, coculturing prevented the transport of NP from the epithelial compartment to the endothelial layer on the bottom of the filter insert. This supports the relevance of coculture models, which favour a differentiated and polarised epithelial layer as *in vitro* test systems for nanoparticle uptake.

1. Background

Nanoparticles (NPs) play a decisive role in industrial applications on the one hand, and on the other hand, NPs are gaining in interest for biomedical research (drug and gene delivery) [1]. Regarding an entry of NPs via inhalation, the alveolar region of the lung with a surface area of 100–140 m^2 make it an interesting target for drug and gene delivery, but at the same time, the lung represents a significant portal of entry for harmful nanomaterials. Inhaled silica nanoparticles (SNPs), for example, embody a serious health-risk characterised by environmental and occupational lung diseases (silicosis) [2]. It has been proposed that pulmonary release of cytokines and mediators into the circulation, that are triggered by inhaled NPs, cause extrapulmonary effects [3]. Epidemiological studies revealed that particulate air pollution (PM_{10}: Particulate matter <10 μm) increased the frequency of cardiac diseases [4,5]. However, plausible explanations from the biological perspective are still lacking. It is also suggested that the resulting systemic

Abbreviations: AmOrSil, amorphous organosiloxane particles; ANOVA, analysis of variance; aSNP, amorphous silica nanoparticles; CatD, cathepsin-D; Cav, caveolin-1; CC, coculture; CHC, clathrin heavy chain; DLS, dynamic light scattering; EC, endothelial cells; EEA1, early endosome antigen 1; Flot1, flotillin-1; Flot2, flotillin-2; IF, immunofluorescence; M6PR, mannose-6-phosphate receptor; MC, conventional monoculture; NP, nanoparticle; Pen/Strep, penicillin/streptomycin; PM10, particulate material with a diameter <10 μm; RFU, relative fluorescent unit; SNPs, silica nanoparticles; TEM, transmission electron microscopy.

* Corresponding author. University Medical Centre, Institute of Pathology, Mainz, Germany. Tel.: +49 (0) 6131 173925; fax: +49 (0) 6131 17534.
E-mail address: kasperj@uni-mainz.de (J. Kasper).

0939-6411/$ - see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.ejpb.2012.10.011

effects are caused by an excess of inhaled PM_{10} that migrate into the systemic circulation and then translocate to different organs [6,7].

Thus, if a lung application is envisaged, toxic effects and the cellular pathways as well as the further disposition of inhaled NPs need to be addressed to gain more insight concerning the above mentioned hypotheses.

Cytotoxicity and cellular uptake/trafficking of nanoparticles in the lower respiratory tract are still poorly understood. One reason for this is that the alveolar-capillary barrier of the deep lung is difficult to access by *in vivo* studies. Therefore, we have inspected nanoparticle interactions on an *in vitro* coculture model of the alveolar-capillary. This *in vitro* model consists of the epithelial cell line, NCI H441 (with characteristics of type II pneumocytes and Clara cells) and the human microvascular endothelial cell (MEC) line, ISO-HAS-1, which are seeded on opposite sides of a transwell filter membrane. Both cell types in coculture (CC) reach a more differentiated and polarised phenotype than if the cells are kept under conventional monoculture (MC) conditions [8,9]. Therefore, it more closely mimics the *in vivo* situation of the deep lung.

Two silica-based nanoparticles such as Sicastar Red (aSNP: amorphous silica, 30 nm in diameter) and AmOrSil (poly(organosiloxane), ca. 100 nm) have been used. Sicastar rather resembles SNPs that are used for industrial purposes and embodies a cytotoxic NP, which is supposed to evoke inflammatory responses to study cell communication processes in the coculture. Whereas AmOrSil is prospectively envisaged for *in vitro* studies concerning drug and gene delivery and is proposed to be nontoxic. AmOrSil has a magnetic core, which may be useful for therapeutic applications (hyperthermia, magnetic resonance imaging or drug delivery) [10,11].

At first, the cytotoxicity (MTS and LDH) was studied on H441 and ISO-HAS-1 in MC and CC. Subsequently, NP uptake behaviour of the epithelial cells (H441) in CC was compared to the epithelial cells kept in MC by fluorescence intensity measurements. Furthermore, transport of NPs across the NP-exposed epithelial layer with subsequent uptake by the endothelial layer (ISO-HAS-1) on the opposite side of the transwell filter membrane was examined. In addition, NP-exposed cells were immunofluorescently counterstained for endosomal marker proteins such as clathrin heavy chain or caveolin-1 as well as flotillin-1 and -2 to examine specific uptake mechanisms such as clathrin-dependent or caveolae-dependent endocytosis.

Finally, the release of inflammatory mediators (IL-8, sICAM) has been examined after NP exposure to the apical side of the coculture (H441) to study inflammatory responses and cell communication processes between epithelial and endothelial cells. In correlation with the uptake/transport experiments with the coculture, these results provide an approach to the hypothesis concerning indirect (forwarded inflammatory mediators caused by NPs) or direct (translocation of NPs) extrapulmonary effects caused by inhaled nanoparticles.

2. Materials and methods

2.1. Nanoparticle characterisation

2.1.1. AmOrSil

AmOrSil nanoparticles were synthesised and delivered by Stefanie Utech (Department of Physical Chemistry of the Johannes Gutenberg University, Mainz). These NPs are magnetic nanocapsules with magnetic iron oxide particles incorporated into a poly(organosiloxane) network that carries an additional PEO shell. The synthesis of the poly(organosiloxane) core–shell nanoparticles was performed in aqueous dispersion by co-condensation of a mixture of alkyldialkoxysilanes (diethoxydimethylsilane) and alkyltrialkoxysilanes (trimethoxymethylsilane and (chloromethylphenyl)-trimethoxysilane, as functional monomers) in the presence of a surfactant. Rhodamine B was covalently incorporated into the entire SiO_x-matrix. Magnetic iron oxide nanoparticles (γ-Fe_2O_3) with an average radius of 3.2 nm were encapsulated during the polycondensation process. Water-solubility was achieved via a grafting-on process, in which linear PEG (poly(ethylene glycol), MW: 1650 g/mol) was covalently attached to the poly(organosiloxane) surface. The magnetic nanocapsules have a primary particle radius of 48.1 nm. Synthesis and characterisation have previously been previously described by Utech et al. [10,11].

2.1.2. Sicastar Red

Sicastar Red is an amorphous silica nanoparticle (30 nm in size) in aqueous dispersion which contains rhodamin B covalently incorporated into the entire SiO_2-matrix. The manufacturing technique is described by micromod Partikeltechnologie GmbH [12].

The hydrodynamic radii of both Sicastar Red and AmOrSil particles in aqueous solutions (water, phosphate buffered saline (PBS) and serum-free cell culture medium RPMI) were determined via dynamic light scattering (DLS) as previously described for the characterisation of non-fluorescent amorphous silica nanoparticles [9]. The results are shown in Table 1. Both samples show an increased hydrodynamic radius in salt-containing media compared to the primary particle radius (determined by transmission electron microscopy and asymmetrical flow field-flow fractionation, data not shown). In the case of the Sicastar Red, the dispersions destabilized with higher salt contents and the particles partly agglomerate; for the AmOrSil, the increase in size compared to the primary particles is not yet completely understood, but it can probably be explained by loose entanglements of the attached poly(ethylene oxide) molecules. The mean hydrodynamic diameter of both particles was ca. 100 nm (radius: 48.1 nm).

2.1.3. Cell culture

ISO-HAS-1 (human microvascular endothelial cell line [13,14]) and NCI H441 (human lung adenocarcinoma cell line, purchased from ATCC, ATCC-HTB-174, Promochem, Wesel, Germany) were grown in RPMI 1640 supplemented with 10% FCS (foetal calf serum), 1% P/S (Penicillin/Streptomycin). ISO-HAS-1 and H441 were passaged every third day at a dilution of 1:3 until passage 50 and 35, respectively.

2.1.4. Monocultures in experimental procedures

Prior to seeding cells, the 96-well plates (TPP, Switzerland) or eight well μ-slides (ibidi) were coated with 50/300 μl fibronectin for 1 h at 37 °C (5 μg/ml, Roche Diagnostics, Mannheim). The cells were seeded (ISO-HAS-1: 1.6×10^4 cells/well, H441: 3.2×10^4 -cells/well) from a confluent culture flask on 96-well plates in RPMI 1640 medium (Gibco) with L-glutamine supplemented with 10% FCS and Pen/Strep (100 U/100 μg/ml) and cultivated at 37 °C, 5% CO_2 for 24 h prior to NP exposure to a confluent cell layer.

Table 1
Hydrodynamic radii of the silica-based nanoparticles (SicaStar Red and AmorSil) in different media obtained via dynamic light scattering.

Medium	H_2O	PBS buffer	Cell medium
AmorSil			
$\langle R_h \rangle_z$ (nm)	52.9	47.9	48.1
μ_2	0.17	0.09	0.11
Sicastar Red			
$\langle R_h \rangle_z$ (nm)	12.6	66.2	58.1
μ_2	0.10	0.16	0.17

Please cite this article in press as: J. Kasper et al., Flotillin-involved uptake of silica nanoparticles and responses of an alveolar-capillary barrier *in vitro*, Eur. J. Pharm. Biopharm. (2012), http://dx.doi.org/10.1016/j.ejpb.2012.10.011