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It takes more than a coating to get nanoparticles through the intestinal barrier *in vitro*

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ABSTRACT

Size and shape are crucial parameters which have impact on the potential of nanoparticles to penetrate cell membranes and epithelial barriers. Current research in nanotoxicology additionally focuses on particle coating. To distinguish between core- and coating-related effects in nanoparticle uptake and translocation, two nanoparticles equal in size, coating and charge but different in core material were investigated.

Silver and iron oxide nanoparticles coated with poly (acrylic acid) were chosen and extensively characterized by small-angle x-ray scattering, nanoparticle tracing analysis and transmission electron microscopy (TEM). Uptake and transport were studied in the intestinal Caco-2 model in a Transwell system with subsequent elemental analysis. TEM and ion beam microscopy were conducted for particle visualization.

Although equal in size, charge and coating, the behavior of the two particles in Caco-2 cells was different: while the internalized amount was comparable, only iron oxide nanoparticles additionally passed the epithelium. Our findings suggest that the coating material influenced only the uptake of the nanoparticles whereas the translocation was determined by the core material.

Knowledge about the different roles of the particle coating and core materials in crossing biological barriers will facilitate toxicological risk assessment of nanoparticles and contribute to the optimization of pharmacokinetic properties of nano-scaled pharmaceuticals.

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1. Introduction

Understanding the interactions between nano-scaled objects and living cells is of great importance for risk assessment [1]. A large number of publications are dealing with the toxicity of nanoparticles, due to their suspected greater toxicity compared to bulk material. It is generally accepted that particle characteristics such as material, size, shape and coating have an influence on particle toxicity [2–6]. Prior to assessing the toxicity of orally ingested particles for target tissues such as the liver, however, it is of interest whether their toxicokinetic properties allow the particles to reach these target cells. Therefore, particles need to overcome some barriers when they are taken up orally: first, they come in contact with the intestinal juices like saliva, gastric and intestinal juice. It has been shown that certain nanoparticles can overcome this gastrointestinal passage and reach the intestinal cells as nano-scaled objects [7–9]. Second, the nanoparticles need to be taken up by the intestinal epithelial and translocate to the basolateral side. Alternatively, they might overcome the epithelial monolayer on a paracellular route.

The intestinal epithelium composed of a tight monolayer of enterocytes constitutes a main part of the gastrointestinal barrier. *In vitro* models like the human adenocarcinoma cell line Caco-2 are available to mimic this barrier. The Caco-2 cell line is able to spontaneously differentiate into enterocyte-like cells forming a polarized monolayer similar to enterocytes *in vivo* and is therefore routinely used as an *in vitro* model of the intestinal barrier [10]. Furthermore, this model can be improved by co-culturing with goblet-like cell lines in order to obtain a model also containing

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the intestinal mucus, or by co-culturing with a lymphocyte cell line to include phagocytosis-specialized M-cells into *in vitro* experimentation.

In many studies, the uptake of particles or substances into Caco-2 cells is used as a surrogate for their systemic bioavailability. This view, however, falls short of reality because transport through the monolayer of enterocytes is a more predictive parameter and often not directly correlated with the amount of particles taken up into the cells. Kenzaoui et al. for example showed a coating-dependent uptake of polyvinylamine- and oleic acid-coated iron oxide nanoparticles into Caco-2 cells, but no transport through the cell monolayer [11]. The transport of nanoparticles through the Caco-2 barrier was only analyzed in comparably few studies [12,13], or as a validation step of the M-cell-model using fluorescent latex beads [14]. As M-cells are specialized on particle uptake, there are studies showing an effect of the surface modification of latex nanoparticles on the transport rate [15]. Nonetheless, one has to bear in mind that translocation of a particle over a barrier of enterocytes *in vitro* does still not fully reflect all steps of gastrointestinal absorption which together determine systemic bioavailability *in vivo*.

Beside the size and shape, by which nanoparticles are defined, the coating material is also of interest as it has an influence on cellular particle uptake and targeting of particles to certain cell types. In most cases, the coating material is necessary for stable nanoparticle dispersion, otherwise particles would agglomerate and aggregate and thereby lose their nano-specific size and properties. Current research in the field thus focuses on the particle coating material as a determinant of particle uptake [4,16,17]. Coating materials have been used to target the particles to for example cancer cells or to improve bioavailability [18–20]. If nanoparticle surfaces are loaded with molecules which adhere to cell surfaces or are normally taken up due to their function in cellular metabolism, the uptake of the particles can be enhanced. This was demonstrated for example with vitamin B12- and different lectin-coated nanoparticles or bioadhesive polymer particles [12,21].

While published literature clearly indicates that cellular uptake is highly dependent on particle coating, it is still not understood to which proportion the coating material of a nanoparticle impacts its transport through cells. This process might still be decisively influenced by particle coating but also by the core material, for example due to the loss of particle coating within a living cell. Therefore, the core material should be kept in focus for determining further fate of the particles. In order to distinguish between core- and coating-related effects in nanoparticle uptake and translocation behavior, two nanoparticles equal in coating, size, shape, and charge but different in their core material were investigated in several Caco-2-based *in vitro* systems of the gastrointestinal barrier with respect to their uptake into intestinal cells and their translocation through the enterocyte monolayer.

2. Materials and methods

2.1. Chemicals

Unless mentioned otherwise, the chemicals used in this study were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), or Carl Roth (Karlsruhe, Germany) at the highest available purity.

2.2. Nanoparticles and characterization

The selection of suitable nanoparticles was based on their comparability regarding their core size, size in biological fluids, shape, etc. As two different particles fulfilling these requirements were not available, poly (acrylic acid) (PAA)-coated iron oxide nanoparticles (fluidMAG-PAS) were purchased by Chemicell (Berlin, Germany) and comparable PAA-coated silver nanoparticles were synthesized using silver nitrate and 1800 g/mol poly (acrylic acid) in a polyol process as previously described by Hu et al. [22]. This approach was followed as previous experience had shown that PAA is suitable to ensure similar characteristics of synthesized nanoparticles with silver or iron as particle core material.

Particle characterization via small-angle X-ray scattering (SAXS) and nanoparticle tracking analysis (NTA) was performed for the stock suspensions of both particles as well as for the diluted particles under cell culture conditions (i.e., following 24 h of incubation in cell culture medium containing 10% fetal calf serum (FCS)) to simulate cell treatment conditions.

SAXS data were acquired using a SAXSess instrument (Anton Paar, Graz, Austria) as described in [7,8]. Background correction of measured intensity was performed by subtraction of the intensity of a solvent solution-filled capillary. Scattering data were deconvoluted (slit length desmearing) after background correction using the software SAXSquant (Anton Paar). SASfit software for curve fitting [23] and curve simulation by Monte Carlo methods with the McSAS software [24] were used for evaluation of the SAXS data. Particle shape was previously checked by transmission electron microscopy (TEM).

NTA was performed at room temperature on a NanoSight LM20 (NanoSight, Amesbury, United Kingdom), equipped with a sample chamber with a $\lambda = 650$ nm laser. Data were captured and analyzed using NTA 2.3 software. Samples were diluted with water down to concentrations of about 10^7–10^9 total particles/mL. Diluted samples were measured three times for 60 s; gain adjustments were performed manually.

Ion release from particles was determined by ultracentrifugation and subsequent determination of silver or iron by atomic absorption spectroscopy (AAS). Cell culture medium was incubated for 24 h with silver or iron oxide nanoparticles in the highest concentration used for experimentation with cell cultures at 37 °C in a humidified atmosphere. Afterwards the medium was centrifuged for 1 h at 100,000*g* (Ultracentrifuge Optima TLX; Beckman Coulter, Krefeld, Germany). One half of the supernatant was collected and digested as described below. The amount of ionic silver or iron in the supernatant was then analyzed by AAS as described below. Additionally, nanoparticles were incubated under acidic conditions thus mimicking the passage through gastric juice following oral uptake, or mimicking lysosomal digestion after cellular uptake of particles. Therefore, 20 μg Ag/ml silver nanoparticles and 200 μg Fe/ml iron oxide nanoparticle were incubated either in pH 3.5 water (acidified with HCl) or in lysosomal fluid pH 4,5 for 24 h, subsequently centrifuged and digested as describes above to examine the intracellular ion release. The composition of the lysosomal fluid is according to Herting et al [25].

Zeta-Potential determination of particles in stock solution (pH 7) was conducted by using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). Nanoparticle stock solutions were diluted to 100 μg/mL and measured ($n = 6$) at room temperature. Additionally, thermogravimetric analysis of PAA-coated silver nanoparticles determined a PAA content of 15% [8] and, as supported by the manufacturer, a PAA content of 10% for the iron oxide nanoparticles.

3. Nanoparticle uptake and transport

3.1. Cell cultivation

Caco-2 cells (ECACC: 86010202) and mucus-producing HT-29 MTX E12 cells (ECACC: 12040401) were obtained from the European Collection of Cell Cultures (Porton Down, UK). Raji-B

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