Silica nanoparticles of microrods enter lung epithelial cells

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Abstract. A novel type of microparticle has recently been engineered. It consists of amorphous silica nanoparticles and has a corncob-like shape. It has already been demonstrated *in vivo* that alveolar macrophages in the lung are able to engulf this particulate carrier and that it also functions successfully as a gene delivery system. This subsequently raises the question as to whether epithelial cells may also be possible targets for these microrods. For this purpose, the alveolar epithelial cell line A549 was used presently. The epithelial character of these confluent cells was documented by the presence of tight junctions using a freeze-fracture technique and transmission electron microscopy. A toxic effect of the particles incubated with these cells was largely excluded. The interaction of the microparticles with the epithelial cells was observed using confocal microscopy and live cell imaging. Interestingly, the particles entered the epithelial cells within hours. After 1 day, the intracellular particles began to disaggregate and release the silica nanoparticles. Thus, even epithelial cells may serve as targets for this novel carrier and gene delivery system. This is particularly important since safe and effective gene delivery remains an unsolved problem. In addition, delivery of anti-cancer and anti-infective drugs may be an application of this novel particulate carrier.

Introduction

Genetic therapy is a prospective strategy for many genetic disorders and diseases. For instance, cystic fibrosis is an important inherited disease in which a specific membrane molecule, the cystic fibrosis transmembrane conductance regulator (CFTR), is absent or not functional (1). The CFTR is a chloride channel and responsible for the chloride concentration and viscosity of secretions (1). However, there remains to be a need for safe and efficient gene therapy carrier systems for different cell types. Recently, a novel carrier system was developed (2) and implemented for the delivery of drugs or plasmids to human macrophages (3). A corncob-like microparticle composed of amorphous silica nanoparticles was created and loaded with a gene construct for luciferase. Those particles could serve as a gene delivery system to phagocytosing cells *in vitro* and *in vivo*; notably, it was demonstrated that the particles were engulfed by professional phagocytes, alveolar macrophages. Days after engulfment of the microparticles, the alveolar macrophages were confirmed to be transfected with luciferase (2). Thus macrophages, as the primary phagocytes, may even engulf these relatively large microparticles of 10 μm in length and 3 μm in width. However, a prevailing issue was whether non-phagocytotic cells could also exhibit carrier uptake. It was therefore investigated in the current study whether the carrier system could be engulfed by epithelial cells. For the current study the human epithelial cell line A549 was selected. This cell line was derived from a lung tumour and reflects properties of the alveolar epithelial type II cells, meaning that the cells grow to a confluent layer *in vitro* (4). In the present study, to confirm the epithelial phenotype of A549 cells, a freeze-fracture electron microscopy technique was employed. Overall the aim of the present study was to clarify whether these non-phagocytic A549 cells could engulf relatively large microrod carrier systems comprised of microparticles. Therefore, to observe the fate of the microrods within the alveolar epithelial cells, different imaging methods were applied: fluorescence microscopy and second confocal laser scanning microscopy. In addition, the viability of the A549 cells following microrod incubation for different times was investigated using the MTT assay.

Materials and methods

Cells and materials. The human epithelial cell line A549 was obtained from Merck KGaA, Darmstadt, Germany. Cells were cultivated at 37˚C in a humidified atmosphere with 5% CO_2 in RPMI-1640 medium with 10% fetal bovine serum (Merck KGaA), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The microparticles were produced from amorphous silica

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nanoparticles (Polysciences GmbH, Eppelheim, Germany) as described previously (2) and had a length of 10 μm and diameter of 3 μm. The microparticles were suspended in aqua dest distilled water, and the number of particles per μl were determined by the use of a Neubauer counting chamber. A total of $2x10^6$ particles were incubated with $1x10^5$ cells to establish a ratio of 20:1. The incubation times with the microparticles were 3, 6, 8, 24 and 48 h.

Fluorescence and confocal laser scanning microscopy. The living cells incubated with microrods were analysed by fluorescence microscopy (Carl Zeiss AG, Oberkochen, Germany) and confocal laser scanning microscopy (VTinfinity, VisiTech, UK), as previously described for mouse cardiac ventricular myocytes (5). Cell borders were labelled using the membrane dye CellMask Deep Red according to the instructions of the supplier (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All microscopy examinations were performed at room temperature (20-22˚C).

Cell viability. For an MTT assay, A549 cells were cultured in 96-well plates with 200 μl RPMI-1640 medium per well. Each well contained $1x10^4$ cells and 0 (control), $1x10^4$ or $1x10^5$ microrods. Following incubation for 1, 3, 6 and 24 h the cells were washed once with sterile phosphate-buffered saline (PBS). The cells were then incubated in a humidified atmosphere of 5% CO_2, 95% air at 37˚C with 5 mg/ml MTT (Merck KGaA) dissolved in PBS buffer at 1:10 ratio under gentle shaking for 4 h. Removal of the MTT reagent was followed by the addition of 100 μl dimethyl sulphoxide and incubation for 20 min in the humidified atmosphere of 5% CO_2, 95% air at 37˚C to dissolve the formazan crystals. To determine cell viability, the absorbance at 550 nm was measured using a microplate spectrophotometer. For each concentration and time point, triplets were used.

Freeze-fracture and electron microscopy. For freeze-fracture analysis, A549 cells were cultured on poly-L-lysine coated glass coverslips. The confluent layer was fixed with 2% paraformaldehyde at room temperature for 5 min. Incubation in 30% glycerol in Soerensen's phosphate buffer (0.15 M, pH 7.4) as pre-vitrificational cryoprotection was performed at 4˚C for 30 min. Small pieces of the glass coverslips were mounted headfirst onto the flat top of copper specimen carriers (Baltic-preparation, Niesgrau, Germany) and subsequently plunge-frozen in nitrogen-cooled liquid ethane using a cryopreparation chamber (Leica Microsystems GmbH, Wetzlar, Germany). Frozen samples were mounted onto a nitrogen-cooled double replica table and inserted into a BAF060 freeze-fracture device (Leica Microsystems GmbH). Freeze-fracturing was performed by cracking the double carrier open at -162˚C and $1x10^{-7}$ mbar pressure. Fractured samples were coated with a 1-nm pre-carbon coat applied at a shadowing angle of 90˚, a 1-nm platinum-carbon coat applied at 60˚ and a second carbon coat of 20 nm applied at 90˚ (carbon coat: carbon rods 3x50 mm, carbon/platinum coat: carbon rods 2x20 mm, platinum inlets 1.5x2 mm; Leica Microsystems GmbH). The frozen replicas were stabilized on a gold index grid (Plano GmbH, Wetzlar, Germany) using a drop of 0.5% Lexan polycarbonate plastic dissolved in dichloroethane (DCE; Acros Organics; Thermo Fisher Scientific, Inc.) (6). To evaporate the DCE and consolidate the Lexan, the assembly of carrier-sample-replica-Lexan -grid was incubated at -20˚C for 16 h. Subsequently, the samples were thawed at room temperature and the carriers were removed. Digestion of the cell samples was performed under agitation in SDS-digestion buffer (2.5% SDS, 10 mM Tris-HCl, pH 8.9) at 60˚C for 27 h. Prior to electron microscopy analysis, the Lexan film was resolved and removed by dipping the replica in DCE. Analysis was performed in a FEI Technai G2 transmission electron microscope (Thermo Fisher Scientific, Inc.) at 100 kV. Pictures were taken with an 8-bit camera at an image size of 1.42 megapixels (Olympus MegaView III; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

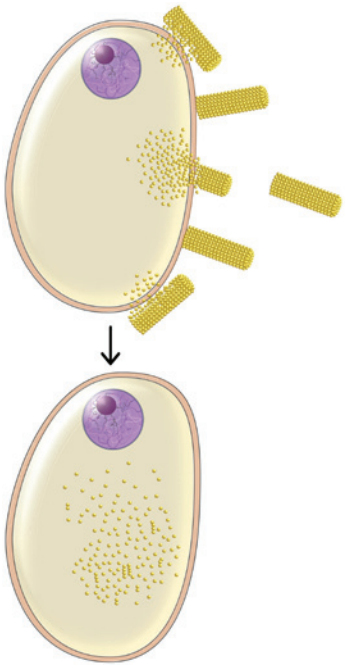

Figure 1. Graphical abstract. A schematic drawing illustrating the entry of microrods into an epithelial cell and their subsequent disaggregation within the cell.

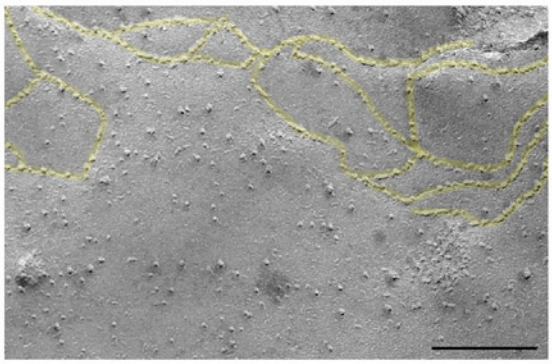

Figure 2. Tight junctions in A549 cell membranes. Freeze-fracture replica of the cell membrane, showing the protoplasmic leaflet (P-face). The formation of tight junction strands is characteristic of epithelial cells and was visible in the form of meandering arrangements of pits and particles (yellow overlay). Magnification, x120,000; scale bar, 100 nm.

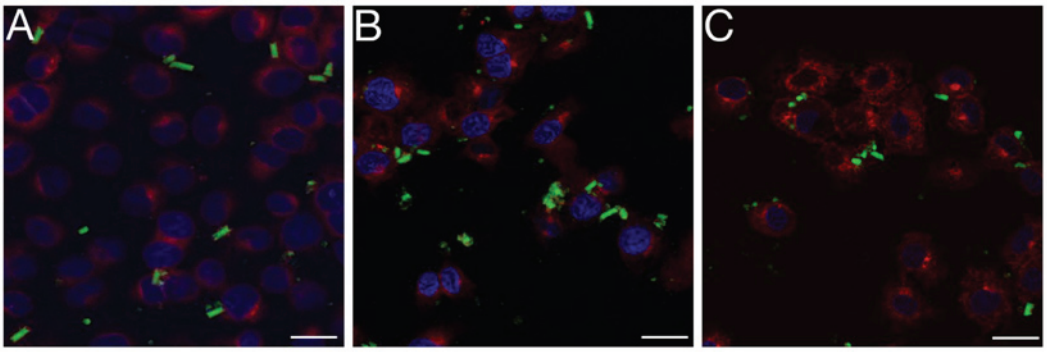

Figure 3. A549 cells and green fluorescent microrods detected by immunofluorescence after (A) 8, (B) 24 and (C) 48 h of incubation. The geometry of the microrods and its relation to the epithelial cells can be visualized. Magnification, x400; scale bars, 40 μm.

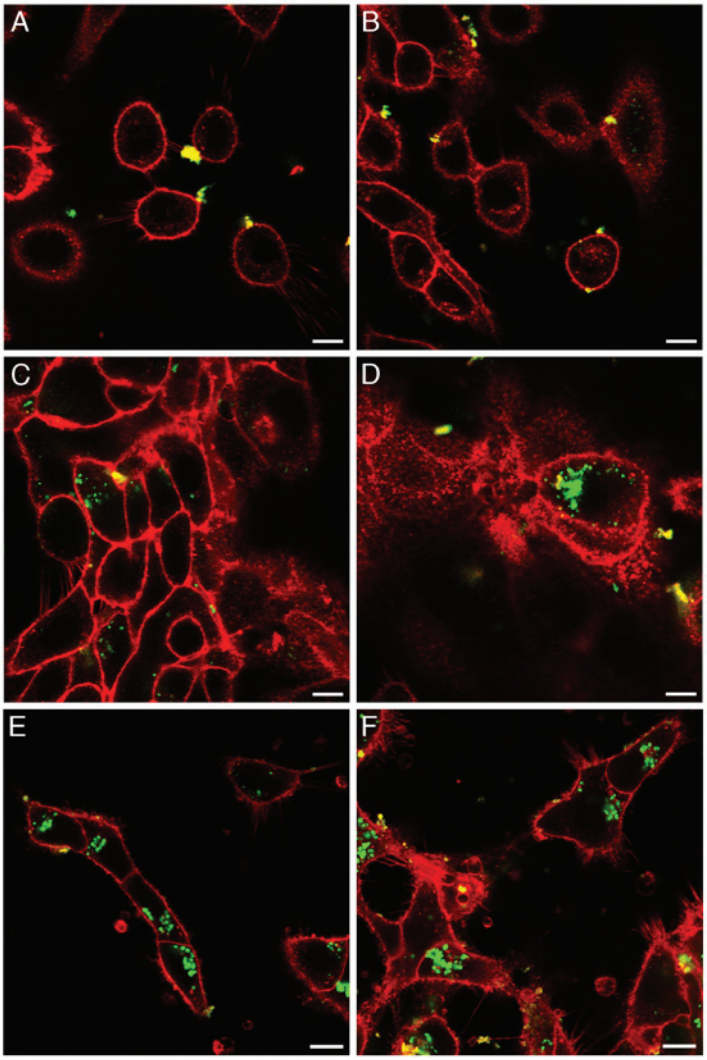

Figure 4. A549 cells (stained with 5μg/ml CellMask Deep Red plasma membrane stain 15 min prior to imaging; red channel) at (A and B) 1 h after the addition of microrods in a ratio of 1:20 (green channel). The microrods appeared attached to the plasma membrane. At (C) 3 and (D) 6 h into the experiment, the microrods were attached to the A549 cells and there was some disassembly to nanoparticle components. Nanoparticles and aggregates of nanoparticles were also observed within the cells. At (E and F) 48 h into the experiment, almost all microrods had disassembled and many nanoparticles were identified within the A549 cells. The nanoparticles appeared to accumulate around the golgi apparatus inside the cells. Magnification, x600; scale bars, 10 μm.

Results

Confirmation of epithelial cell character. It was uncertain whether the microrods are able to enter epithelial cells. The A549 cell line used is established as an epithelial cell line, which was supported by the freeze-fracture analysis (Fig. 2). In this representative image, distinct tight junctions are apparent.

Effects on viability. From the MTT assay, cell viability at a 1:1 ratio of cells to microparticles was 101.9±0.9% after 1 h, 99.9±3.7% after 3 h, 103.6±1.5% after 6 h, 95.9±5.6% after 24 h, and at a 10:1 ratio was 103.7±5.1% after 1 h, 94.0±3.2% after 3 h, 90.3±2.5% after 6 h and 83.6±2.1% after 24 h (data not shown).

Imaging. The fluorescence microscopy provided visualisation of the epithelial cells following co-incubation with the microrods at different time points (Fig. 3). After 8 h, microparticles remained extracellular (Fig. 3A). After 24 h, the majority of the rods had started to disaggregate (Fig. 3B). After 48 h, nearly all of the rods appeared to have disaggregated, and nanoparticles or conglomerates of nanoparticles appeared to be intracellular (Fig. 3C). Through confocal analysis, this process could be observed in greater detail and higher resolution (Fig. 4). After 1 h, attachments of the rods to the cells were observed (Fig. 4A and B). After 3 and 6 h (Fig. 4C and D), disaggregated nanoparticles were observed within the cells. After 48 h (Fig. 4E and F), many epithelial cells contained nanoparticles.

Discussion

The intention of this short report was to highlight that this novel drug delivery system may not only reach macrophages but also enter epithelial cells. The corncob-like microrods have previously been reported to be engulfed by macrophages and to successfully transfect macrophages (2); however, due to their dimensions, they were considered incapable of entering epithelial cells. The epithelial cells used were A549 cells. These cells were originally derived from a lung carcinoma and share many features with type II alveolar epithelial cells (3). In particular, the cells are characterised by fast replication and confluential growth (4). To confirm the epithelial character of these cells, a freeze-fracture technique was employed presently, in order to visualize tight junctions, the presence of which is characteristic for epithelial cells and tissues. The toxic effects of the microrods were generally excluded by determining the viability of the cells. However, a notable reduction of viability (<90%) was observed after 1 day of incubation at a particle-to-cell ratio of 10:1. Conventional fluorescence and confocal laser scanning microscopy were used to describe the transitional process undertaken by the microrods and provided spatial and temporal resolution. Following their initial adhesion to the A549 cells, the rods disaggregated to silica nanoparticles and these appeared to penetrate the cell membranes and enter the cytoplasm. The A549 cells are a useful cell line since they possess some remnants of alveolar type II cells, including the capacity for the synthesis of lamellar bodies (4). These cells may possibly also have certain endocytotic properties. Admittedly, inhibitors of specific endocytosis pathways that could have detected phagocytotic mechanisms (7) were not tested.

A potential application of this microrod-based approach is the targeting of human bronchial and intestinal cells. In this regard the rods could be used to carry transcripts for a range of genetic disorders. One example would be the CFTR to rescue hereditary mutation in this molecule. This may be a viable option to treat patients with cystic fibrosis, as a common hereditary disease. A long-term aim is to reach epithelial basal cells or stem cells, in order to further target and potentially cure genetic disorders including cystic fibrosis.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TT, MS and TF planned the study. TF prepared the microrods and performed the MTT assay. CM, AB and AG performed the freeze-fracture, electron microscopy and interpretation of the corresponding results. AG performed the cell culture and fluorescence imaging. PL and AG performed the laser scanning confocal microscopy. TT was a major contributor in the writing of the manuscript. All authors interpreted data, and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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