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Research Paper

Macrophage uptake of cylindrical microparticles investigated with correlative microscopy

Clemens Tscheka [a,1], Marius Hittinger [b,1], Claus-Michael Lehr [b,c], Nicole Schneider-Daum [b], Marc Schneider [a,c,*]

[a] Pharmaceutics and Biopharmacy, Philipps University, Marburg, Germany
[b] Drug Delivery, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI), Saarbrücken, Germany
[c] Department of Pharmacy, Biopharmaceutics and Pharmaceutical Technology, Saarland University, Saarbrücken, Germany

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ABSTRACT

Cylindrical particles offer the opportunity to develop controlled and sustained release systems for the respiratory tract. One reason is that macrophages can phagocyte such particles only from either of the two ends. We investigated the uptake behaviour of murine alveolar macrophages incubated with elongated submicron-structured particles. For that purpose, fluorescent model silica nanoparticles were interconnected with the biocompatible polysaccharide agarose, building up cylindrical particles within the pores of track-etched membranes. In contrast to common approaches we determined the uptake at different time points with scanning electron microscopy, fluorescence microscopy, and the combination of both techniques – correlative microscopy (CLEM). As a consequence, we could securely identify uptake events and observe in detail the engulfment of particles and confirm, that phagocytosis could only be observed from the tips of the cylinders. CLEM allowed a comparison of the uptake measured with different techniques at identical macrophages. Qualitative and quantitative evaluation of this cylindrical particle uptake showed substantial differences between fluorescence microscopy, electron microscopy and the combination of both (CLEM) within 24 h.

1. Introduction

Shape of micro- and nanoparticles is increasingly gaining attention because it has been revealed that the geometry alters fundamental properties. Interactions with biological systems differ for non-spherical particles, opening up new options for the design of drug delivery systems [1–3].

Alveolar macrophages clear the respiratory region from foreign materials including pathogens and senescent cells. This physiologically highly relevant task, essential for homeostasis of healthy tissue and clearance, was found to be strongly influenced by the shape of the object [1,2]. Modification of the geometry changes the time and mechanism required for uptake [3]. As a consequence, non-spherical particles such as cylindrical particles have the potential to control clearance processes, a core prerequisite for a sustained release system for therapeutics [2,4].

Fibres and cylinders show a higher probability to deposit in the deep lung, which is beneficial for pulmonary administration, in comparison with spheres of identical volume [5]. Cylinder-like particles can be prepared following various techniques [2]. Tailor-made truly cylindrical particles require bottom-up formation within a template, dictating the geometry. These techniques include the fabrication approaches such as PRINT [6], polymerization in microfluidic devices [7] and the template technique [2]. The template technique is the only approach that has been reported to allow for the formation of highly ordered (in close-packing of spheres fashion) sub-structured cylinders, composed of nanoparticles [8]. Track-etched membranes with uniform cylindrical pores serve as templates for the formation of these hierarchical cylindrical microparticles in high fidelity. The cylinders are composed of silica nanoparticles that are coated with the biocompatible polysaccharide agarose.

In order to assess the uptake, we incubated murine alveolar macrophages with these cylindrical particles and determined the particle uptake utilizing correlative light and electron microscopy (CLEM). Electron microscopy (EM) is the technique of choice, when high magnification and fast image acquisition are required. The high resolution reveals details invisible for any light microscopic

* Corresponding author at: Biopharmaceutics and Pharmaceutical Technology, Saarland University, Campus A4 1, D-66123 Saarbrücken, Germany. Tel.: +49 81 302 2438; fax: +49 681 302 4677.
E-mail address: Marc.Schneider@mx.uni-saarland.de (M. Schneider).
[1] These authors contributed equally.

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technique. The advantage of fluorescence light microscopy (FLM) is the high specificity. Correlative microscopy combines the information on the very same position of a sample, offering more insight than the single techniques [9]; it is not the statistical comparison of huge populations of different entities, permitting to securely observe even rare events in detail. We analyzed identical positions of fixed macrophages interacting with elongated particles. Differences between kinetic studies based on the single approaches and the correlated use of FLM and SEM to our knowledge are being reported for the first time.

2. Methods

2.1. Preparation of cylindrical particles with submicron texturing

For the preparation of the cylindrical particles, an adapted procedure derived from the template-assisted polyelectrolyte (PE) encapsulation of nanoparticles protocol [8] was applied. The polyelectrolytes PAH (polyallylamine hydrochloride) and PSS (polystyrene sulphonate) were replaced by the natural polysaccharide agarose. Polycarbonate (PC) track-etched membranes with a thickness of approximately 10 μm and a pore size of 2 μm (Nuclepore Track-Etched Membrane, 25 mm, Whatman, Dassel, Germany) were utilized as a template for the formation of the cylindrical particles. For the bottom-up approach, plain blue fluorescing silica beads (λ_{ex} = 354 nm/λ_{em} = 450 nm, Kisker Biotech GmbH & Co. KG, Steinfurt, Germany) with a diameter of 500 nm were filled into the void space of the membrane. The arrangement of close-packing of equal spheres of the beads gives rise to the submicron texture of the cylinders. A solution of 1.5% (100 °C) agarose (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was used for the interconnection of the spherical particles in order to conserve the cylindrical geometry given by the template. Being in liquid state, the agarose solution could enter the pores and interconnect the silica particles. Thereafter, the template membrane was dissolved in tetrahydrofuran (THF) (Tetrahydrofuran AnalaR NORMAPUR, VWR International GmbH, Darmstadt, Germany) and the particles were subsequently purified by centrifugation. After the last cycle the pellet was redispersed in RPMI-medium (5% FCS, 1% Penicillin/Streptomycin). The particles redispersed well without aggregation. Hence, this allowed to optically determine the particle concentration in the stock solution using a Neubauer chamber. For application the suspension was diluted to a final concentration of 100,000 particles/ml in RPMI-medium (5% FCS, 1% P/S).

2.2. Uptake experiment and sample preparation

20,000 murine alveolar macrophages (MHS, ATCC, CRL-2019) per plate were cultured for 24 h on glass plates (22 × 22 mm, Paul Marienfeld GmbH, Lauda-Koenigshofen, Germany) in RPMI 1640-medium (5% FCS, 1% P/S) (PAA, Pasching, Austria) containing standard supplements. Then, the growth medium was changed with medium containing 100,000 cylindrical particles per plate. To analyze the uptake profile, cells were fixed at different time points after addition of particles (0, 1.5, 3, 4.5 and 24 h). For fixation, cells were incubated in 100% methanol (VWR International GmbH, Darmstadt, Germany) for 10 min and washed three times in phosphate buffered saline (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Afterwards, they were incubated in 3% glutaraldehyde (Merck KGaA, Darmstadt, Germany) for two hours, and dehydrated with increasing alcohol concentrations (Ethanol 70%, 80%, 95%, 100%, 100%; exchange rate: 1 h; VWR International GmbH, Darmstadt, Germany) and air-dried. The samples were sputtered with a gold layer of ~15 nm thickness prior to CLEM imaging (Sputter coater: Quorum Q150R ES, Quorum Technologies Ltd, East Grinstead, UK). For each sample, several randomly selected frames were captured using FLM until a count of 300 ± 20 macrophages was reached.

2.3. Visualization with CLEM

The Shuttle & Find™ extension (Carl Zeiss Microscopy GmbH, Jena, Germany) permits a straightforward relocation of any region of interest (ROI) with its standardized sample holder, both in the FLM (Axio Imager M1m, equipped with the LED system Colibri for excitation, Carl Zeiss Microscopy GmbH, Jena, Germany) and in the SEM (EVO HD15, Carl Zeiss Microscopy GmbH, Jena, Germany), not requiring any manipulation on the sample for relocation. Each image (ROI) is saved with its coordinates. Usually FLM is performed as first analysis, because the electron beam could corrupt the fluorophores [9]. The fluorescence was excited at λ_{ex} = 365 nm for the cylindrical particles and at λ_{ex} = 470 nm for the cells; bandpass filters 445/50, 525/50 respectively were used for the emitted light. Afterwards, the holder was transferred to the complementary device and calibrated again. SEM imaging was carried out using 5 kV acceleration voltage and the secondary electron (SE) detector. Images of the two microscopes were then superimposed with the provided software.

3. Results and discussion

The cylindrical particles were well-dispersed, both in THF and in buffer, and did not aggregate permanently, or change in other relevant respects; this could be seen during counting in the Neubauer chamber and the SEM analysis respectively. The cylinders were highly uniform, resembling the inverse features of the template (2.0 × 10 ± 1 μm) in high fidelity, with a length of 10.24 ± 1.47 μm (RSD 14.4%) and a width of 1.99 ± 0.08 μm (RSD 3.91%) (n = 27). Ruptured or deformed particles were rare. SEM analysis also reveals the highly ordered arrangement of the silica nanoparticles in a close-packing of spheres fashion that has been preserved by the interconnecting agent agarose. The silica particles serve as a model for hydrophilic nanoparticles [8]. Independent of the surface material (agarose with its hydroxyl moieties or silica) the cylinders' surface will be hydrophilic, contributing to the stable suspension observed. However, the preparation strategy is derived from the template-assisted interconnection with polyelectrolytes [8] in which the core particles are completely covered. This suggests that the agarose fully envelops the beads.

A concise analysis of the uptake behaviour could be undertaken based on high resolution technique SEM. The engulfment of cylindrical particles was observed to take place from the ends without exception (Fig. 1), supporting the paradigm that phagocytosis is highly orientation and shape dependent [1,2,4]. This means that the geometry at the point of first contact between the macrophage and the particle governs the initiation of phagocytosis. The curvature of a sphere represents the threshold for uptake. If the curvature of the structure is too little, the phagocyte merely spreads on the objects and continues scouting for high curvature regions [1], such as the tips. After identification of these geometries internalization is initiated. This behaviour is displayed in the CLEM image Fig. 1(a) representing two stages of internalization. The onset of internalization can be seen for the particle laying tangential and touching the cell with one end. Its cell membrane extends over the tip, whereas no invagination can be seen from the long axis. The intermediate state of uptake can be seen with the second particle in Fig. 1(a), half has already been internalized with one end being closest to the phagocyte. Further insight into the biochemical process could be achieved for example with an actin-staining [1]. The completion of the uptake process is dictated by the size of