

# **Macrophage uptake of cylindrical microparticles investigated with correlative microscopy**

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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 phagocytose such particles only from [either of the two ends](#). We investigated the uptake behavior 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 hours.

## **Keywords**

Fluorescence Light Microscopy (FLM), Scanning Electron Microscopy (SEM), Correlative Light and Electron Microscopy (CLEM), microparticles from nanoparticles, shape dependent uptake, non-spherical particles, phagocytosis

## 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].

Fibers and cylinders show a higher probability to deposit in the deep lung, which is beneficial for pulmonary administration, in comparison to 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 is required. The high resolution reveals details invisible for any light microscopic 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 analysed 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 are being reported to our knowledge reported for the first time.

## **Methods**

### **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 sulfonate) were replaced by the natural polysaccharide agarose. Polycarbonate (PC) track-etched membranes with a thickness of approximately 10  $\mu\text{m}$  and a pore size of 2  $\mu\text{m}$  (Nucleopore 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

( $\lambda_{\text{ex}} = 354 \text{ nm}/\lambda_{\text{em}} = 450 \text{ nm}$ , 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).

### **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 hours). For fixation, cells were incubated in 100 % methanol (VWR

International GmbH, Darmstadt, Germany) for 10 minutes 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 hour; 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  $300 \pm 20$  macrophages was reached.

### **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 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  $\lambda_{\text{ex}} = 365 \text{ nm}$  for the cylindrical particles and at  $\lambda_{\text{ex}} = 470 \text{ nm}$  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.

## Results & 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 ( $2.0 \times 10 \pm 1 \mu\text{m}$ ) in high fidelity, with a length of  $10.24 \pm 1.47 \mu\text{m}$  (RSD 14.4 %) and a width of  $1.99 \pm 0.08 \mu\text{m}$  (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.

**Fig. 1.** Phagocytosis of cylindrical particles. CLEM images displaying the selective invagination of submicron structured cylindrical particles (blue) from **the ends** by murine alveolar macrophages. The CLEM images consist of the SEM micrograph on which the fluorescence signal of the particles from the FLM image is superimposed. The fluorescence signal (blue) of the cylindrical particles results from the labelled silica beads. **(a) The SEM micrograph with higher magnification reveals more details, here the invagination of the tip of the particle.**

A concise analysis of the uptake behavior 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. 1a) 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. 1a), 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 the particle and is limited by the**



volume of the phagocyte [1]; the dimension of particles was selected permitting uptake by the phagocytes.

Taking into consideration the texturing of the cylindrical particles composed of spherical submicron particles (500 nm), the observed behaviour is surprising at first glance. The local shape encountered by the macrophage is the morphology of spheres, representing a high curvature shape, embedded into the matrix in a close-packed fashion. Nonetheless, no engulfment could be observed from the flat side most likely due to the fact that the substructure and its protrusion is too little. This might also be due to the swelling of the connecting agarose gel in aqueous media, which can mask the underlying structure of the sphere, rendering it too minute to still be sensed by the macrophages.

The phagocyte has to move around the cylindrical particle in order to achieve an adequate orientation for complete invagination, approaching from either of the two ends. [This shape-induced delay of internalization extends the residence time of the carrier system, representing a new design parameter for the retardation of clearance \[4\].](#) The nature of the used particles dictates the conditions of release, either within the phagocyte or before internalization. [Changing the material and composition of the particles used for cylinder preparation should allow tailoring the release profile for the desired purpose. This shape and orientation dependent uptake also translates into a systematic error for quantification with FLM \(Fig. 4\).](#) The uptake for all time points is in general overestimated in comparison to the more precise CLEM, because the resolution does not in all cases permit to differentiate between tangential adherence of the cylinders and already completed internalization (Fig. 2).

**Fig. 2.** Correlation of typical fluorescence and SEM images. Macrophages are shown by auto fluorescence (bright yellow) and particles were fluorescently labeled (blue). The FLM image indicates two uptake processes in the selected region (a). Judging from the electron microscopy image, no particle uptake could be observed (b). The correlated image (c) allows distinguishing between engulfed particles (macrophage on the left) and those which are only in contact with cells (particle on the right).

**Fig. 3.** Uptake process of a cylindrical particle in detail. (a) In the FLM image (400 × magnification) possible interactions between particle (blue) and macrophage can be identified (dashed circle). (b) The overlay of SEM and FLM images shows an ongoing uptake process. (c, d) Further SEM images allow a clear examination of the process of internalization of particles, revealing that the particle was nearly completely engulfed here. *The plasma lemma has progressed over most of the particle, only approximately 1 μm of the cylinder in length is not yet invaginated. The borders of the plasma membrane are indicated by arrows.*

For the determination of the uptake kinetics cells were fixed at various time points and engulfed particles quantified with FLM and SEM separately. Besides the stand-alone techniques the data was merged using the overlay of the two images. No discrimination of initiated or completed ingestion was performed; these scenarios were interpreted as uptake.

Analyzing the macrophage uptake with correlative microscopy can avoid three different scenarios of misinterpretation and consequently a bias in the kinetics. Firstly, particles adhering to the cell membrane that have not been engulfed can be easily identified with SEM, whereas analysis with FLM is much more prone to false interpretation (box in Fig. 2a) and Fig. 2b), right). This leads to a massive overestimation of the internalization kinetics for early time points based on solely FLM analysis (Fig. 4). The bias levels off during the time course of the experiment. After longer incubation more and more adhering particles are taken up, diminishing the misinterpretation by FLM. This shape dependent uptake mechanism [1] extends the internalization time. Secondly, particles which are completely invaginated cannot be seen with the aid of SEM but can securely be detected with fluorescence microscopy (Fig. 2c), left). Therefore, SEM-based kinetics suffer from increased discrepancy in comparison to CLEM. Over time an increasing number of the particles is concealed by the cell membrane and hence cannot be identified with the electron microscope. The third case comprises the ongoing process of phagocytosis. This cannot be seen explicitly with FLM and should further be investigated with SEM (Fig. 3). Overall, the comparison of the stand-alone techniques reveals the benefits of the analysis with CLEM, resulting in more precise uptake values. Furthermore, we can compare FLM and SEM based kinetics with the corrected value of correlative microscopy within the same studied macrophages (Fig. 4). However, for non-phagocytes or even other particles, it is conceivable that a cell is simply covering a particle without internalization. The SEM visualizes only the surface and fluorescence microscopy is limited by its projection which in this case leads to false-positive results. FIB-SEM, which allows for a controlled milling of the sample, could be applied and complement CLEM for other cases [10].

**Fig. 4.** Uptake kinetics of cylindrical particles by murine alveolar macrophages. The graph illustrates the difference of the uptake kinetics utilizing FLM, SEM and correlative microscopy (CLEM) determined on the same fixed macrophages. The values are given in percent and represent the number of cells that have partially or completely invaginated at least one particle over the total count of cells in the images captured for the study ( $n = 300 \pm 20$  macrophages for 3 independent experiments). The exact value (%) is given over the respective bar. The black bar shows the uptake based on FLM, the grey bar the uptake based on SEM and the dark grey bar the correction by CLEM. The error bars indicate the standard deviation for 3 experiments.

With all techniques we could observe an increase of particle uptake over time (Fig. 4). The standard deviations for the 3 quantification approaches based on 3 independent experiments are lower for early (1.5 h) and late time points (24 h) of incubation. Variations of experimental conditions alter the activity of cells and therefore the internalization velocity which translates into discrepancies between the experiments in particular for intermediate time points. After long incubation all objects are taken up, diminishing the difference. The uptake based on FLM was more than doubled within 24 h compared to the SEM studies. The number of particles taken up is well described by SEM in the beginning (< 6 h) of the experiments, whereas FLM is more suited for later time points. If reliable data for the whole uptake kinetic is needed, correlative microscopy is the best choice.

The cellular uptake behavior is strongly dependent on the size and shape of particles as well as on the used cell type; this is reflected in different time courses for each set of parameters. Overall, correlative microscopy is the most suited technique for time points during the uptake process, in our case below 24 h. For later time points after uptake has occurred, FLM or similar techniques are also well-suited. In addition, correlative microscopy using the Shuttle & Find™ system can overcome obstacles such as the time consuming finding of different regions in both microscopes. In the future, CLEM may develop towards a useful tool to further elucidate uptake kinetics for a variety of particles and different cell types avoiding misleading results.

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## Figure Captions

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