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Polymer particle shape independently influences binding and internalization by macrophages

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ABSTRACT

The interaction of macrophages with micro and nanoparticles (MNPs) is important because these cells clear particles from the circulation, and because they are potential therapeutic targets in inflammatory conditions, atherosclerosis and cancer. Therefore, an understanding of the features of MNPs that influence their interaction with macrophages may allow optimization of their properties for enhanced drug delivery. In this study, we show that particle shape impacts phagocytosis by macrophages, and more importantly, that particle shape and size separately impact attachment and internalization. The study provides a methodology for further exploring how particle shape can be controlled to achieve desired attachment and internalization. The results of the study also give mechanistic guidance on how particle shape can be manipulated to design drug carriers to evade macrophages, or alternatively to target macrophages.

1. Introduction

Polymeric micro- and nanoparticles (MNPs) have considerable potential for use in drug delivery, vaccination and imaging [1–3]. The interaction between MNPs and macrophages, cells that specialize in the internalization of foreign particles, is of interest for two reasons. Macrophages of the reticuloendothelial system (RES) enact rapid clearance of particles from the circulation [4,5], a process that limits the ability of MNPs to extend the circulating half-life of a drug. Knowledge on how to design particles that escape phagocytosis would help to overcome this limitation. One the other hand, macrophages are frequently mobilized in response to disease and play a central role in inflammation, infectious disease, tumor growth, and atherosclerosis [6–10]. Consequently, these macrophages are viable drug targets, and in these cases knowing how to design particles to enhance uptake by macrophages is of benefit. Accordingly, the objective of this study was to develop an understanding of how the shape of MNPs influences the attachment and internalization by macrophages during phagocytosis.

Macrophages internalize foreign particles by phagocytosis, a process in which particles attach to, and are then engulfed by the plasma membrane. This draws the particles into the cell to form an internal phagosome [11], where the particle can be degraded. Prior work has shown that phagocytosis is influenced by the size and surface chemistry of the foreign particle [11–16]. For example, particles of an intermediate size (~2 μm) are phagocytosed more rapidly than smaller or larger particles [13,17,18]. Alternately, particles with hydrophobic surface are phagocytosed more rapidly than particles with hydrophilic surfaces [13,19]. Additionally, surface modification of particles by neutral hydrophilic polymers such as polyethylene glycol (PEG) can also influence the rate of phagocytosis [15,16].

The influence of particle shape on phagocytosisis is only beginning to be studied. Convenient methods to fabricate particles with different shapes were only recently developed [12,20,21]. Champion and Mitragotri reported a method for preparing particles of various shapes by stretching spherical particles [12]. Using such particles, they found that phagocytosis, at a single particle level, depends on the local particle shape. However, the impact of particle shape on phagocytosis at an ensemble level has not been studied. Here, we quantify the effect of shape on phagocytosis of an ensemble of particles. We also, for the first time, measure how shape influences the independent processes of attachment and internalization, which together define the phagocytic rate.

The study uses ellipsoids as a model non-spherical particle because the aspect ratio of ellipsoid particles can be easily controlled during fabrication, allowing one to generate particles with a wide range of curvatures. Furthermore, ellipsoid particles accumulate at the vessel wall during laminar flow of blood, so these particles are likely to be more suitable for vascular targeting than spheres [22–24]. We also chose to focus on the process of phagocytosis, as opposed to pinocytosis or endocytosis, because phagocytosis is largely responsible for the uptake of larger particles, which can encapsulate more drug.

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2. Methods

2.1. Particles

Spherical polystyrene particles (0.5–3.6 μm in diameter) loaded with fluorescent dye (Fluospheres) were purchased from Invitrogen (Carlsbad, CA). Polyvinyl alcohol (PVA) was purchased from Sigma (St. Louis, MO). Procedures used to manufacture particles of different shapes were similar to those developed by Champion and Mitragotri. Briefly, 10% PVA was dissolved in hot water (75 °C) containing 2% glycerol to increase plasticity, and spherical particles were added at a concentration of 0.04% w/v. The solution was poured into a custom 19×25-cm flat tray and left to dry and form a thin film. The film was then stretched either in 1 dimension (1D) or in 2 dimensions (2D) using custom-made devices. The aspect ratio of the particles was determined by the length of stretching imposed on the film. Films were stretched at 120 °C in oil (above the glass transition temperature of PS). Stretched films were cooled at room temperature and soaked in isopropyl alcohol for 24 h to remove any residual oil. All films were dissolved in 30% isopropyl alcohol/water at 65 °C and washed 10 times by centrifugation to remove all PVA from the surface of the particles. The finished particles were characterized by using a scanning electron microscope (Philips XL30 ESEM) and Multisizer-3 coulter counter (Beckman Coulter, Fullerton, CA).

2.2. Macrophage cell culture

The RAW264.7 monocyte/macrophage mouse cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). They were cultured in DMEM media containing L-glutamine and sodium pyruvate (Mediatech, Manassas, VA), supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin.

2.3. Confocal microscopy

RAW264.7 cells were grown in 8-well tissue culture-treated slides (BD Biosciences) at a concentration of 1×10^5 cells/ml, incubated at 37 °C for 24 h. Non-attached cells were removed by aspiration and 200 μl of fresh media containing 1×10^7/ml MNPs was added per well and returned to 37 °C. The next day, the cells were fixed with 4% PFA, washed with PBS and incubated with phalloidin:Alexa 488 in 0.1% Tween-20/PBS to stain polymerized actin filaments. After washing twice with 0.1% Tween-20/PBS, the cell nuclei were stained with DAPI and the slides mounted and sealed. To obtain three-dimensional images of cells associated with particles, cells were incubated with particles of different shapes containing a red dye (ex580/em605). Following a 4 h incubation, cells were fixed, cytoplamic actin filaments were stained with phalloidin:Alexa 488 (green) and nuclei were stained with DAPI (blue). The position of MNPs within the cell was gauged with confocal optical z-section, and cross-sectional images.

2.4. Assessing phagocytosis with flow cytometry

RAW264.7 cells were plated at 5.8×10^5 cells/well in 6-well non-tissue cultured plates (BD Biosciences, Sparks, MD) for 24 h (37 °C, 5% CO_2, humidified). Particles suspended in 0.5 ml of culture media were then added to each well giving a final cell to particle ratios of 1:40 (0.5 μm), 1:10 (1 μm) and 1:1.1(3.6 μm). The cells were collected at different time points by detaching them from wells using 0.05% trypsin and ethylenediaminetetraacetic acid (EDTA). Aggregates of cells were disrupted by vigorous pipetting. The resuspended cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffed saline (PBS) for 5 min, stained with either rat anti-mouse CD68:FITC (Serotec, Raleigh, NC), phalloidin Alexa:488 (Molecular Probes, Eugene, OR), or rabbit anti-alpha tubulin (Abcam, Cambridge, MA) followed by a goat anti-rabbit IgG:FITC. After washing twice in PBS, the number of particles associated with cells was analyzed by flow cytometry with a FACSCanto (BD Biosciences). For each experiment, 10,000 FITC-positive events were collected and analyzed using FACSDiVa software.

2.5. Quantifying the independent steps of phagocytosis (attachment vs. internalization)

To independently measure the role of attachment and internalization of MNPs we used a previously reported method to dissolve particles external to the cell with xylene, while leaving internalized particles intact [25]. Particles were allowed to bind to macrophages for 4 h at 4 °C, a condition under which internalization doesn't occur [26,27]. The number of particles attached to the macrophage surface was then quantified using fluorescent microscopy by counting 500 macrophages on approximately two-third of the area on each slide. Sister samples, washed in the same manner, were shifted to an incubator at 37 °C to allow internalization of the attached particles. The internalization process was stopped after 1 h by fixing the cells with 4% PFA. The PFA-fixed macrophages were air-dried, then immersed in xylene at room temperature for 10 min with gentle agitation to dissolve the extracellular polystyrene particles. Xylene was then evaporated and cells were stained with phalloidin:Alexa 488. Confocal images of nanoparticles associated with cells were taken before and after the xylene treatment to verify that the process removes external particles while preserving those within the cell (see Supplemental Fig. S1).

3. Results

3.1. Fabrication of particles of different shape

MNPs of three different shapes were produced by stretching the spherical fluorescent polystyrene (PS) spheres, using previously described methods [12]. The stretching procedures were scaled-up so that particle size and morphology could be maintained constant across all studies reported herein. Spheres of three different diameters were used for stretching: 0.52 μm (volume = 0.075 μm^3), 1.0 μm (volume = 0.69 μm^3) or 3.6 μm (volume = 24.42 μm^3). Three shapes were generated (Fig. 1): spheres (radius 0.26–1.8 μm), prolate ellipsoids (major axis 0.35–2.5 μm, minor axis 0.2–2 μm), and oblate ellipsoids (major axis 0.35–2.5 μm, minor axis 0.2–2 μm).

3.2. Phagocytosis of particles by macrophages

To obtain indications of particle phagocytosis we took a series of confocal z-section images of MNPs incubated with RAW264.7 mouse macrophages for 4 h at 37 °C. Confocal images showing z-sections and the corresponding fluorescent/brightfield overlay were obtained for particles of each shape (Fig. 2). Each series of panels represents

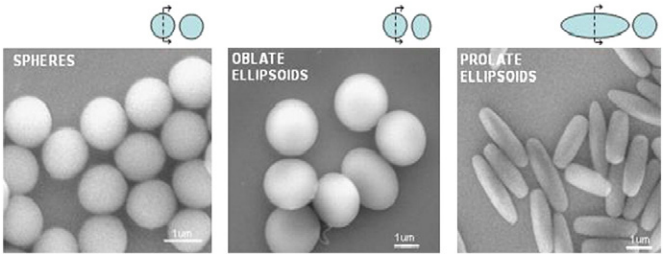

Fig. 1. SEM images of the particles used in this study. Particles of different shape were created from spherical particles using 1D and 2D stretching methods according to procedures set forth in Methods.

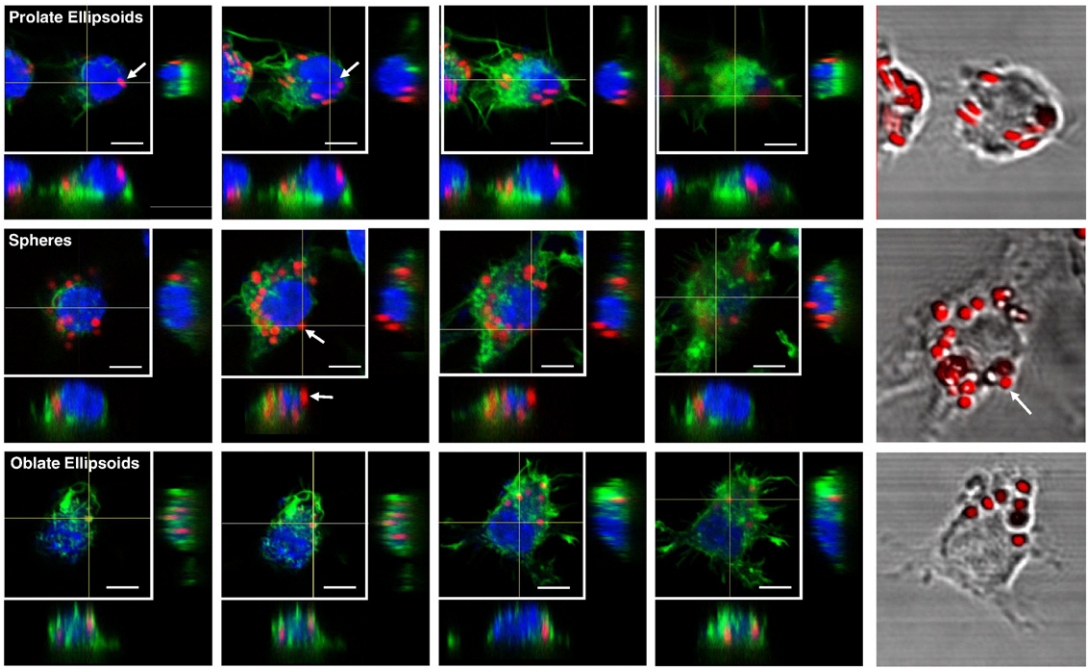

Fig. 2. The association of 1 μm-sized (volume = 0.69 μm^3) particles with cells after incubation for 4 h was visualized by confocal microscopy. Cells were incubated with particles of different shapes which contained a red dye (580ex/605em). Following particle association, cells were fixed and actin filaments were stained with phalloidin:Alexa 488 (green) and nuclei were stained with DAPI (blue). Confocal optical z-section images taken through the top of the cell surface to the bottom (from Left to Right) are shown. For the cross-section image, the large panel is the x–y section, the small panel at the bottom is the x–z section, and the small panel on the right is the y–z section. Rightmost panel shows the corresponding fluorescence/brightfield overlays. Arrows indicate particles that appear to be cell surface associated and not internalized. Scale bar = 5 μm.

images taken in the x–y plane moving from the top of the cell to the bottom (Fig. 2, left to right). In this series of images, particles associated with the cell surface are observed in the initial panel, or at the edges of cells in subsequent panels. Particles that are inside cells are observed in the middle panels in close proximity to actin and the nuclei. Cross-section images of cells were taken at the cross-hair in the x–z plane (bottom panel) and in the y–z plane (side panel, right). Two examples of particles that are surface bound but not internalized are noted with arrows. The prolate ellipsoid on the surface is evident in the confocal image representing the top of the cell, and also at the top of the x–z plane. The particle is no longer evident in sequential images which show the interior of the cell. Similarly, one of the spherical particles on the edge of a cell is noted with arrows in the full confocal image, in the x–z plane, and also in the brightfield image. All other particles appear to be within the interior of the cell.

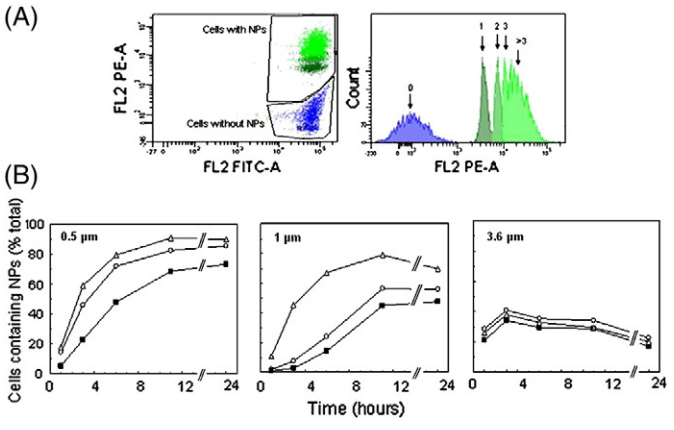

Fig. 3. (A) Evaluation of particle phagocytosis with FACS. Cells were visualized in the FITC channel while cells containing red fluorescent particles were visualized in the PE channel (*Left panel*). For larger particles, populations with one, two and three particles could be clearly seen (*Right panel*). (B) Association of particles with macrophages was quantified; spheres (○), prolate ellipsoids (■) and oblate ellipsoids (△). The number in the upper left corner of the graphs represents the diameter of the original sphere from which the particles were generated. In these experiments standard deviations are about 5% of the mean and have been omitted for clarity.

Fluorescence activated cell sorting (FACS) was used to extend the analysis and quantify the extent of phagocytosis of each type of particle (Fig. 3A). Interestingly, cells containing different numbers of particles could be distinguished by FACS based on the mean fluorescence intensity of the cell (Fig. 3A, right panel, arrows). However, to compare overall phagocytosis, across all sizes of particles, we simply scored the percentage of cells that had phagocytosed one or more particles. In these experiments, the number of particles added to macrophages was normalized according to total particle volume. In other words, the cells were challenged with the same total volume of particles. In all cases, phagocytosis reached saturation by 11 h (Fig. 3B).

For the smallest particles (derived from spheres of 0.5 μm), oblate ellipsoids exhibited a slightly higher level of phagocytosis when compared to spheres (Fig. 3B, left panel). Both of these shapes were phagocytosed more efficiently than prolate ellipsoids. A similar trend was observed with particles derived from 1 μm spheres, except that in this case oblate ellipsoids were phagocytosed more efficiently than either spheres or prolate ellipsoids. In fact, at early time points (~2 to 6 h) the association of oblate ellipsoids was more than double that of the other shapes (Fig. 3B, middle panel). Altogether these observations reveal clear differences in the phagocytosis of particles with different shapes, although these differences are apparent only in particles with small and intermediate sizes; differences were not evident with the larger particles derived from spheres of 3.6 μm in diameter (Fig. 3B, right panel).

3.3. Independent measures of cell binding and internalization

Phagocytosis is a combination of two separable parameters: 1) the attachment of particles to the macrophage surface, and 2) the rate at which these bound particles are internalized. Studies were conducted to determine which of these parameters is influenced by particle

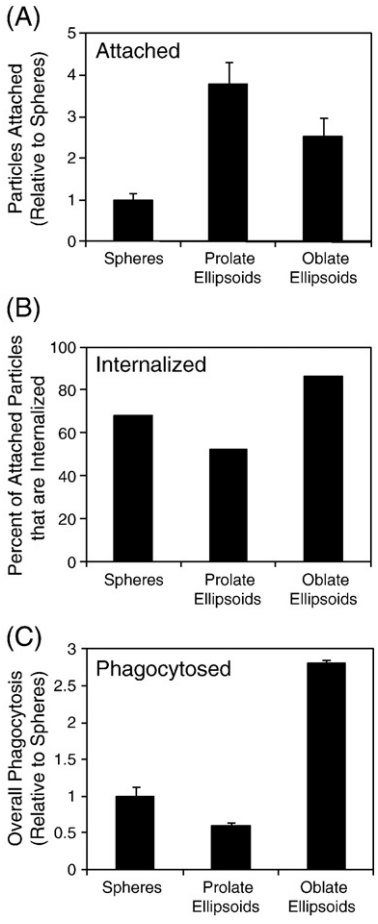

Fig. 4. Comparison of attachment and internalization of particles of various shapes prepared from 1 μm spheres. (A) Number of particles attached to cells; (B) Particles internalized by cells and (C) Overall phagocytosis after a 6 h incubation (re-plotted from Fig. 3B).

shape. Particles derived from spheres of 1 μm diameter, were used for this analysis. Attachment was measured by incubating particles with macrophages at 4 °C for 4 h and quantifying the association using fluorescent microscopy. Sister samples of cells were washed to remove unattached particles and then shifted to an incubator at 37 °C to allow internalization of the attached particles. The internalization process was stopped after 1 h by fixing the cells with 4% PFA and then treating the samples with xylene to dissolve the non-internalized particles as described in Methods. Internalized particles were counted by using fluorescence microscopy.

The attachment of particles to macrophages is ranked such that prolate ellipsoids > oblate ellipsoids > spheres (Fig. 4A). In contrast, the internalization of the attached particles shows a different ranking, where oblate ellipsoids > spheres > prolate ellipsoids (Fig. 4B). Hence, prolate ellipsoids show the best attachment, but the poorest internalization. In fact, only 52% of the attached prolate ellipsoidal particles were subsequently internalized within an hour. In contrast, 86% of the attached oblate ellipsoidal particles were subsequently internalized (Fig. 4B).

The results in Fig. 4A and B show that while prolate ellipsoids attach most efficiently, their probability of internalization is the lowest. The product of these two processes determines the extent of phagocytosis, as shown in Fig. 4C (re-plot of the 6 hour time point of from Fig. 3B). So, the limited phagocytosis of prolate ellipsoids can be explained by the fact that their poor internalization overrides their superior attachment. Oblate ellipsoids, on the other hand, exhibit both high attachment and high internalization and consequently the highest phagocytosis. Altogether, these findings show that shape independently influences particle attachment and internalization by macrophages.

4. Discussion

The salient conclusions of this study are that: (i) the shape of a particle has a profound impact on phagocytosis by macrophages; phagocytosis of oblate ellipsoids is much greater than for prolate ellipsoids or spheres, (ii) the shape of a particle influences cell attachment and internalization independently, (iii) the attachment of particles to macrophages can be ranked in the following order: prolate ellipsoids > oblate ellipsoids > spheres, and (iv) once attached though, internalization of particles follows a different rank: oblate ellipsoids >> spheres > prolate ellipsoids. These conclusions hold for particles with volumes in the range of 0.075 to 0.69 μm^3. No significant difference in phagocytosis is observed for particles with volume of 24.42 μm^3.

Our finding that ellipsoid particles attach more efficiently to the macrophage surface compared to spherical particles is consistent with earlier reports that show particles with longest dimensions close to 2–3 μm exhibit highest attachment [28]. The attachment of particles is certainly necessary for internalization since macrophage activation, and hence particle internalization, is generally known to depend on the particle dose [29]. Our observations, that prolate ellipsoids attach better than spheres, but are phagocytosed less efficiently indicate that attachment is only one aspect of the phagocytic process. This finding further suggests that geometry of a particle can inhibit internalization, a conclusion entirely consistent with recent work by one of our groups showing that particle geometry strongly impacts its internalization by macrophages [12]. Since phagocytosis is an actin-based process [11,30,31], the dependence of phagocytosis on particle shape can be potentially linked to the extent of actin remodeling required by the cellular cytoskeleton in order complete internalization. Since actin remodeling is an energy intensive process [32], shapes that require more actin remodeling should, in theory, be internalized slower.

The fact that oblate ellipsoids are internalized more efficiently than prolate ellipsoids can probably be attributed to their lower aspect ratio, which would require less actin remodeling for internalization. It is also conceivable that oblate ellipsoids indirectly promote their own internalization by activating macrophages, which is reported to depend on the particle dose [29] and geometry [33,34]. So, it is possible that oblate ellipsoids, by their high attachment, could stimulate the macrophage internalization process. The contribution of this and other effects to the observed differences between various geometries needs further investigation.

Studies such as those reported here can guide the rational design of MNPs to achieve the desired attachment and internalization. For example, particles with at least one extended axis, like prolate ellipsoids, are likely to be well suited for use as long circulating drug carriers because their phagocytosis by macrophages in the liver will be inefficient. This idea is consistent with a recent work showing that particles with high aspect ratios are less likely to undergo phagocytosis. Specifically, Geng et. al. [35] studied worm-like micellar particles (filomicelle) possessing very high aspect ratios (AR > 23) and found that longer filomicelles exhibit reduced phagocytosis with the longest filomicelles showing almost no phagocytosis. Similarly, Champion et. al. [36] showed that worm-like polymeric particles possessing high aspect ratios (AR ~ 20) are not phagocytosed. There is also evidence showing a rank-order preference for particles with high aspect ratios in escaping phagocytosis [37]. In contrast to prolate ellipsoids, oblate ellipsoids are likely to be well suited in circumstances where one would like to deploy particles with high uptake by macrophages. For example, oblate ellipsoids might be useful drug-delivery vehicles in conditions like leishmania [38], AIDS [6], and atherosclerosis [39], where macrophages are a therapeutic target.

Altogether, this study provides mechanistic guidance and methodology for further exploration of how particle shape can be manipulated to achieve desired attachment and internalization. Obviously, the range of particle shapes that could be explored is much larger than that studied here, but the general principles uncovered here should still apply. The methodology of separating attachment from internalization can also be of interest to virologists seeking to modulate the infectivity of viruses. Future studies should also focus on extending the findings of this study to other types of cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.jconrel.2010.07.116.

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