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Modeling receptor-mediated endocytosis of polymer-functionalized iron oxide nanoparticles by human macrophages

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ABSTRACT

Although systemically applied nanoparticles are quickly taken up by phagocytic cells, mainly macrophages, the interactions between engineered nanoparticles and macrophages are still not well defined. We therefore analyzed the uptake of diagnostically used carboxydextran-coated superparamagnetic iron oxide nanoparticles of 60 nm (SPIO) and 20 nm (USPIO) by human macrophages. By pharmacological and in vitro knockdown approaches, the principal uptake mechanism for both particles was identified as clathrin-mediated, scavenger receptor A-dependent endocytosis. We developed a mathematical model of the uptake process that allows determination of key parameters of endocytosis, including the rate of uptake, the number of nanoparticles per cell in saturation, the mean uptake time, and the correlation between the number of internalized nanoparticles and their extracellular concentration. The calculated parameters correlate well with experimental data obtained by confocal microscopy. Moreover, the model predicts the individual and collective wrapping times of different nanoparticles, describes the relation between cytoskeletal forces, membrane elasticity and the uptake time. We also introduced a new physical parameter '*a*' governing the collective uptake process, *a* reflecting minimal linear spacing between simultaneously acting neighboring endocytotic pits.

1. Introduction

Due to their unique size-dependent properties, nanoparticles are often indispensable as technical materials, as probes for cell and subcellular structure labeling, as well as for drug and gene delivery. Apart from their well established industrial application in catalytic and separation processes, magnetic nanoparticles are frequently used in a variety of biological and medical applications, such as magnetic resonance imaging (MRI), drug delivery and studies of cell mechanics [1,2]. Most of these applications require well defined and controlled interactions between nanoparticles and living cells. However, our understanding of interactions of nanoparticles with living cells, as well as the uptake mechanisms of manufactured nanoparticles remains rather limited [3,4].

Significant progress has been made in understanding how the geometry of nanoparticles might influence their endocytosis rate [4–6]. However, other characteristics of the particle surface, such as size and coating also affect their cellular uptake. In addition, nanoparticles may elicit various intracellular responses, which can modulate their cellular uptake. Therefore, the cell type, the differentiation state, the levels of receptor expression, and the polymerization state of the actin/myosin cytoskeleton would inevitably influence the uptake mechanisms and their efficacies [4]. Wrapping time is a mathematical parameter integrating the different forces acting on a particle and characterizing the particle uptake. The wrapping time depends on variables, such as particle size and shape, receptor expression, and elasticity of the cell membrane [4]. Because of its inherent complexity, a quantitative description of the process of nanoparticle uptake still remains elusive, with certain key aspects lingering as yet unexplained, without any in vivo validation. Hence, a description of the cellular uptake mechanics of nanoparticles by a mathematical model, taking into account their size and shape, cell membrane elasticity, and the number of expressed receptors would significantly advance our understanding of these dynamic processes. In particular, it would allow better definition of nanoparticle–cell interactions and therefore

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better biomedical applications including drug delivery and hyperthermia [2,7–10].

Superparamagnetic iron oxide particles (SPIO) are nano-sized particles of ferrite used as contrast agent for MRI. As particulate matter, they are taken up by the reticulo-endothelial system (RES), which consists mainly of macrophages [11]. Very small particles of less than 30 nm in diameter (USPIO) remain intravascular for a prolonged period of time and, thus, can serve as blood pool agents.

Resovist (SHU 555A) is a SPIO contrast agent that causes a signal decrease in T_2-weighted MR images. It contains a crystalline core composed of Fe_3O_4 (magnetite) and γ-Fe_2O_3 (maghemite) covered with carboxydextran [12,13]. After intravenous injection of the recommended dose of 0.7 mmol (10 μmol Fe/kg) the blood levels reach 200–300 μmol Fe/l [14]. Within 30 min after injection, Resovist is taken up by liver and spleen, where it is sequestrated by phagocytic macrophages [12,14].

The USPIO Supravist (SHU 555C) is a positive enhancing blood pool contrast agent, which underwent phase III clinical testing [15,16]. The chemical composition of Supravist particles is very similar to that of Resovist, yet due to a thinner carboxydextran shell, their total diameter is smaller giving lower values for the T_2 relaxivities [13,16].

Macrophages are involved in the coordination of immune responses, in the elimination of pathogens, and the control of tissue homeostasis. These multifunctional cells act as scavengers to clear the body of damaged cells, cell debris, various particles, viruses, and bacteria [17]. Macrophages are professional phagocytes that not only possess the ability to engulf particulate matter, but also to express diverse receptors, which facilitate particle uptake by specifically binding to opsonins, mainly antibodies and complement factors [3]. Macrophages also produce a range of proinflammatory cytokines and mediators crucial for the orchestration of innate and adaptive immune responses in inflammation and tissue repair [17].

We have previously shown that human macrophages efficiently take up SPIO and USPIO [13]. The uptake kinetics of iron oxide contrast agents as a function of particle size, shape, surface, as well as the uptake mechanisms has hitherto not been well characterized nor quantified. Therefore, the aim of this study was to investigate the uptake mechanism of SPIO and USPIO by human macrophages and to develop a quantitative mathematical model describing the mechanics of this process.

2. Materials and methods

2.1. Characterization of the particles

The SPIO Resovist™ was obtained from the pharmacy of the University hospital, whereas the USPIO Supravist™ was a gift as stated in the acknowledgments. The average size, the electrophoretic mobility, the zeta potential and the surface charge density of the particles were measured in 0.9% NaCl using a Zetasizer Nano (Malvern Instruments) and have previously been described [13].

2.2. Cell differentiation and treatment

Macrophages were differentiated from human monocytes isolated from buffy coats by density gradient centrifugation using Histopaque 1077 [18]. To induce differentiation, the monocytes were treated every other day with 15 ng/ml M-CSF (R&D Systems). The cells were characterized by flow cytometry and used at day 7 for the treatment with the particles.

Macrophages (1×10^6 cells/ml RPMI 1640, 10% FCS) were pre-incubated with different pathway inhibitors (scavenger receptor, 10 μg/ml polyinosinic acid for 30 min [19]; macropinocytosis, 2 μM rottlerin for 30 min [20]; pinocytosis, 100 μg/ml colchicine for 2 h [21]; phagocytosis, 10 μg/ml cytochalasin B for 2 h [21]; clathrin-mediated endocytosis, 200 μM monodansyl cadaverine for 10 min [22]; caveolae-mediated endocytosis, 50 μg/ml nystatin for 15 min [23]), and treated with the indicated particle concentrations for different time periods.

2.3. Transmission electron microscopy

Macrophages were fixed in Karnovsky's fixative (2.5% glutaraldehyde, 3% paraformaldehyde, 0.1 M sodium cacodylate, pH 7.4), postfixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Epon (Fluka). The ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (EM400, Philips).

2.4. Measurement of intracellular iron

Macrophages 1×10^6 cells seeded in 1 ml RPMI 1640, 10% FCS, were exposed to the nanoparticles for different time periods and different concentrations. Then the cells were washed, dehydrated at 110 °C overnight and iron was dissolved in 100 mM citric acid. Fe^{3+} was reduced to Fe^{2+} with ascorbic acid and treated with bathophenanthroline disulfonic acid. Iron bathophenanthroline disulfate was analyzed spectroscopically [24] using $FeCl_2$ for the generation of standard curves.

2.5. Spinning disk confocal microscopy

Nanoparticles were fluorescently labeled with Alexa Fluor488 hydrazide according to the manufacturer's instruction (Invitrogen). Macrophages seeded on IBIDI slides (Munich, Germany) were treated for 1 h with either SPIO or USPIO (each 500 μg Fe/ml), cell membranes were stained with CellMask™ Deep Red (red dye, Invitrogen). In some experiments, cells were fixed with 4% paraformaldehyde, permeabilized, stained with an antibody against clathrin (clathrin HC, sc-9069, Santa Cruz Biotechnology) and visualized with anti-rabbit $F(ab')_2$ conjugated to Cy5 (Dianova, Hamburg, Germany). Fluorescence images were taken with the acquisition software Andor iQ 1.6 using a custom-designed spinning disk confocal microscope consisting of a CSU10 scan head (Yokogawa, Tokyo, Japan), an inverted microscope (Axio Observer, Zeiss) with an oil immersion objective (UPlanSApo 60×/1.35, Olympus), an environmental control (PECON, Erbach, Germany), an image splitting unit (OptoSplit II, Cairn Research, Faversham, UK) and an EMCCD camera (DV-887, Andor, Belfast, UK). ImageJ software (NIH) was used for image processing, 3D and 5D reconstruction. The number of nanoparticles taken up by a cell was analyzed using ImageJ software (NIH) applying the quantification method described by Vukojevic et al. [25].

2.6. Knockdown of scavenger receptors type A

For in vitro knockdown of scavenger receptor A phosphorothioate-modified oligodeoxynucleotides (ODN) (ThermoHybaid, Ulm, Germany) were used. The ODN were selected on the basis of the major predicted secondary structures, i.e. the loops [26]. The antisense ODN used to downregulate the scavenger receptor A expression corresponds to the nucleotides 738–757 of scavenger receptor A mRNA (NM_004039): 5′-AATAAGTTTGAATACCACA-3′ and targets all splice variants of the receptor. The control sequence contained the same set of the base pairs in a scrambled order. Both sequences were analyzed for lack of secondary structure and oligo pairing. According to Blast search, the selected sequences did not show any similarity to any other mRNA sequence. Macrophages were treated for 48 h every 24 h with 10 μM of the ODN [27]. The cells were either lysed and analyzed for scavenger receptor A expression [27] using an antibody against scavenger receptor A (R&D Systems), or incubated with SPIO or USPIO (each 500 μg/ml) for 6 h, and the particle uptake was analyzed using bathophenanthroline disulfonic acid as described above.

2.7. Statistical analysis

Quantitative results are expressed as mean ± SEM. Results were analyzed by Tukey's or Newman–Keuls multi-group comparisons tests. Differences were considered statistically significant at $*p < 0.05$.

3. Results

3.1. Uptake of SPIO and USPIO by macrophages

Analysis of the uptake of SPIO and USPIO nanoparticles by human monocyte-derived macrophages by transmission electron microscopy revealed that, within 60 min, both nanoparticles were localized within vesicles of about 100–120 nm. There were more iron-loaded vesicles in the macrophages treated with SPIO as compared to USPIO-treated cells (Fig. 1). In line with this observation, macrophages accumulated significantly more SPIO (24.10 ± 0.63 pg Fe/cell) than USPIO (2.30 ± 0.14 pg Fe/cell, $p < 0.01$) (Fig. 2); this was ~60 and 6 times higher than the amount of iron in untreated control cells (0.41 ± 0.23 pg/cell).