Acta Biomaterialia 9 (2013) 8454–8465

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Endocytotic uptake of iron oxide nanoparticles by cultured brain microglial cells

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ARTICLE INFO

Article history:
Received 27 October 2012
Received in revised form 17 May 2013
Accepted 21 May 2013
Available online 31 May 2013

Keywords:
Endocytosis
Iron
Lysosomes
Microglia
Nanoparticles

ABSTRACT

Microglia are the phagocytotic cells of the brain that respond rapidly to alterations in brain homeostasis. Since iron oxide nanoparticles (IONPs) are used for diagnostic and therapeutic applications in the brain, the consequences of an exposure of microglial cells to IONPs are of particular interest. To address this topic we have synthesized and characterized fluorescent BODIPY®-labelled IONPs (BP-IONPs). The average hydrodynamic diameter and the ζ-potential of BP-IONPs in water were ~65 nm and −49 mV, respectively. Both values increased after dispersion of the particles in serum containing incubation medium to ~130 nm and −8 mV. Exposure of cultured rat microglial cells with BP-IONPs caused a time-, concentration- and temperature-dependent uptake of the particles, as demonstrated by strong increases in cellular iron contents and cellular fluorescence. Incubation for 3 h with 150 and 450 μM iron as BP-IONPs increased the cellular iron content from a low basal level of ~50 nmol iron mg^{-1} to 219 ± 52 and 481 ± 28 nmol iron (mg protein)$^{-1}$, respectively. These conditions did not affect cell viability, but exposure to higher concentrations of BP-IONPs or for longer incubation periods severely compromised cell viability. The BP-IONP fluorescence in viable microglial cells was co-localized with lysosomes. In addition, BP-IONP accumulation was lowered by 60% in the presence of the endocytosis inhibitors 5-(N-ethyl-N-isopropyl)amiloride, tyrphostin 23 and chlorpromazin. These results suggest that the rapid accumulation of BP-IONPs by microglial cells is predominantly mediated by macropinocytosis and clathrin-mediated endocytosis, which direct the accumulated particles into the lysosomal compartment.

1. Introduction

Iron oxide nanoparticles (IONPs) are used for neurobiological applications, including cancer treatment by hypothermia, as contrast agents for magnetic resonance imaging (MRI) as well as for targeted drug delivery and cell transfection [1,2]. Direct access of IONPs to brain tissue is achieved by injection into the affected brain area for treatment of brain tumours [3]. However, IONPs that are administered peripherally by oral application, intravenous injection or by inhalation have been reported to enter the brain by crossing the blood–brain barrier or via the olfactory system [1,2,4–6].

Microglial cells are the immune competent cells of the brain. Depending on the situation, microglial cells can be beneficial or harmful to their neighbouring cells. In the healthy adult brain, so-called "resting" microglia survey their microenvironment for nutrients or debris, release neurotrophic factors and anti-inflammatory cytokines, and promote synaptic plasticity [7,8]. However, upon activation by brain injury or infections, microglial cells migrate to the site of the impact and secrete inflammatory proteins and reactive oxygen species (ROS) that may damage neighbouring cells [7,9]. Microglial cells will encounter nanoparticles that have entered the brain, since these cells are known to literally scan their surroundings for debris and particles which are subsequently taken up [7–9]. Indeed, exposure of animals with IONPs as contrast agents for MRI revealed that in the brain especially the microglial cells are strongly labelled [10–13].

Metal-containing nanoparticles (NPs), such as IONPs, titan dioxide NPs, gold NPs, alumina NPs or quantum dots, have been reported to affect microglial functions in vivo and have been connected with cell toxicity, microglial activation, production of ROS and cytokine release [10–18]. However, little information is so far available on the mechanisms involved in the uptake of IONPs

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http://dx.doi.org/10.1016/j.actbio.2013.05.022

by microglial cells. A few studies have used microglial cell lines as model systems to gain information on the consequences of an exposure of microglial cells with IONPs [12,19–22]. However, it has to be considered that the advantageous feature of immortality of cell lines may be accompanied by properties and behaviours that differ from those of primary cells [8,23].

For previous studies on the accumulation of IONPs by cultured brain cells, we used non-fluorescent dimercaptosuccinate (DMSA)-coated IONPs [24,25]. However, visualization of the presence of such particles in cells is difficult and requires electron microscopy. This limitation can be bypassed in part by using fluorescent IONPs for uptake studies; this allows us to obtain information on the intracellular localization of the accumulated IONPs by fluorescence microscopy. As a tool for such studies, we have synthesized fluorescent BODIPY®-labelled IONPs (BP-IONPs) by coating IONPs with BP-labelled DMSA and have characterized these particles for their physicochemical properties and their colloidal stability. With the BP-IONPs generated, we were able to apply high concentrations of IONPs to cultured cells for detailed iron accumulation studies and could also investigate the cellular localization of the accumulated BP-IONPs by fluorescence microscopy.

Cells in secondary microglial cultures have been shown to accumulate fluorescent IONPs by analysis of their cellular fluorescence [13,26]. However, to our knowledge, no detailed quantitative analysis of IONP uptake into microglial cells or identification of mechanisms involved in particle uptake have been reported so far. Here we show by quantitative iron determination, cytochemical iron staining and fluorescence microscopy that cultured primary microglial cells efficiently accumulated BP-IONPs in a time-, concentration- and temperature-dependent manner by endocytotic processes, which direct the accumulated particles into the lysosomal compartment.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS), trypsin solution and penicillin/streptomycin solution were obtained from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Karlsruhe, Germany) and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) from Roth (Karlsruhe, Germany). Bovine serum albumin and nicotinamide adenine dinucleotide were from Applichem (Darmstadt, Germany). BODIPY® FL C_1-IA [N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl) iodoacetamide] and lysotracker Red DND-99 were purchased from Invitrogen (Darmstadt, Germany). 5-(N-ethyl-N-isopropyl)amiloride (EIPA), tyrphostin 23, ferrozine, dimercaptosuccinic acid (DMSA), 4′,6-diamidino-2-phenylindol hydrochloride (DAPI) and paraformaldehyde were purchased from Sigma–Aldrich (Steinheim, Germany). Mouse anti-rat CD11b (Ox-42) antibody was purchased from Serotec (Düsseldorf, Germany) and the Cy3-conjugated anti-mouse immunoglobulin from Dianova (Hamburg, Germany). Other chemicals of the highest purity available were purchased from Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). 96-well microtitre plates and 6-well cell culture plates were from Nunc (Wiesbaden, Germany) and 24-well cell culture plates from Sarstedt (Nümbrecht, Germany).

2.2. Synthesis and characterization of BP-IONPs

IONPs were synthesized by chemical co-precipitation of ferrous and ferric iron salts as described previously [27]. The nanoparticles were coated with DMSA or BODIPY® (BP)-labelled DMSA according to a modification [24] of a published method [28]. BP-DMSA was synthesized by thoroughly mixing 712.5 μM BODIPY® FL C_1-IA and 4.75 mM DMSA at pH 10 in 10 mM NH_3 or in 47.5 mM glycine/NaOH buffer. A 30 min incubation at room temperature (RT) led to the complete derivatization of BODIPY® FL C_1-IA with thiol groups of DMSA (Fig. S.1). Electrospray ionization mass spectrometry revealed the expected signals at 470 and 759 m/z for DMSA labelled with BP on one or both thiol groups, respectively (Fig. S.1). IONPs were added to the BP-DMSA reaction mixture to a final concentration of 21.4 mM and the mixture was acidified to pH 3 by concentrated HNO_3. After mixing for 30 min at RT, the particles were separated from the solution by magnetic force, resuspended in H_2O and redispersed by increasing the pH value with NaOH to pH 9–10. Finally, the pH was lowered to 7.4 by adding HCl. This dispersion was diluted with water to a final iron concentration of 40 mM and stored at 4 °C. Fourier transform infrared spectroscopy confirmed the presence of the coating material in the BP-IONPs generated (Fig. S.2). The concentrations of BP-IONPs used in the individual experiments are given here as concentrations of the iron present in the nanoparticle dispersion and do not represent the concentration of particles.

Samples for transmission electron microscopy (TEM) were prepared by dropping 5 μl of 1 mM BP-IONP dispersion in water onto carbon-coated copper grids and subsequent air drying at RT. Images were taken by a FEI Tecnai F20 S-TWIN (Hillsboro, Oregon, USA) operated at 200 kV and equipped with a GATAN GIF2001 SSC-CCD camera. Energy-dispersive X-ray analysis (EDX) was used for elemental analysis in the scanning mode of the microscope (STEM) with an EDAX r-TEM-EDX-detector with an energy resolution of 136 eV. The hydrodynamic diameters and the ζ-potentials of 1 mM BP-IONPs dispersed in different media were determined at 25 °C by dynamic and electrophoretic light scattering in a Beckman Coulter (Krefeld, Germany) Delsa™ Nano C particle analyser at scattering angles of 165 and 15°, respectively. The fluorescence spectra of diluted BP-IONP solutions (50 μM in water) were recorded using a Cary Eclipse fluorimeter (Varian, Darmstadt, Germany).

The hydrodynamic diameter, ζ-potential and the fluorescence intensity of the dispersed BP-IONPs did not change during storage for at least up to 1 month, nor was release of any low molecular weight iron from the particles detectable during storage (data not shown).

2.3. Cell cultures

Primary microglial cultures were prepared from astroglia-rich primary cultures by tryptic removal of the astrocyte layer using a modification of a published method [29]. Astroglia-rich primary cultures were prepared from the whole brains of neonatal Wistar rats [30] and 300,000 viable cells were seeded per well of a 24-well plate with or without coverslips in 1 ml culture medium (90% DMEM, 10% FCS, 20 U ml^{-1} of penicillin G and 20 μg ml^{-1} of streptomycin sulfate) or 1.5×10^6 cells per well of a six-well plate in 2.5 ml medium. The cultures were grown in a cell incubator (Sanyo, Osaka, Japan) that contained a humidified atmosphere of 10% CO_2/90% air and the culture medium was renewed every seventh day. To obtain microglial cultures, confluent 14- to 23-day-old astroglia-rich cultures were incubated for 30 min with 0.5% (w/v) trypsin in serum-free DMEM. This treatment resulted in the detachment of an intact top layer of cells that contain virtually all the astrocytes and left a population of firmly attached microglial cells in the wells. The microglial cells were washed with 2 ml culture medium and cultured in 1.5 ml glia-conditioned medium (GCM; 0.2 μm filtered glia-conditioned culture medium harvested after 1 day of incubation of astroglia-rich primary cultures) for additional 16–20 h before experiments were performed. The cultures obtained by this method are highly enriched in microglial