Toxicology Letters 225 (2014) 57–65

Contents lists available at ScienceDirect

Toxicology Letters

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

Interaction of polyacrylic acid coated and non-coated iron oxide nanoparticles with human neutrophils

Diana Couto[a], Marisa Freitas[a], Vânia Vilas-Boas[b], Irene Dias[a], Graça Porto[c], M. Arturo Lopez-Quintela[d], José Rivas[e], Paulo Freitas[e], Félix Carvalho[b], Eduarda Fernandes[a,*]

[a] REQUIMTE, Laboratory of Applied Chemistry, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal
[b] REQUIMTE, Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Porto, Portugal
[c] Service of Clinical Hematology, Santo António Hospital, Porto, Portugal
[d] Laboratory of Nanotechnology and Magnetism, Institute of Technological Research, IIT, University of Santiago de Compostela (USC), Santiago de Compostela, Spain
[e] International Iberian Nanotechnology Laboratory, Braga, Portugal

HIGHLIGHTS

- Polyacrylic acid-coated iron oxide nanoparticles increase neutrophils' apoptosis.
- Non-coated iron oxide nanoparticles prevent neutrophils' apoptosis.
- Both nanoparticles trigger neutrophils' oxidative burst by NADPH oxidase activation.

ARTICLE INFO

Article history:
Received 30 August 2013
Received in revised form
17 November 2013
Accepted 18 November 2013
Available online 26 November 2013

Keywords:
Nanotoxicology
Oxidative burst
Apoptosis
Human neutrophils
Iron oxide nanoparticles
Polyacrylic acid coating

ABSTRACT

Iron oxide nanoparticles (ION), with different coatings and sizes, have attracted extensive interest in the last years to be applied in drug delivery, cancer therapy and as contrast agents in imagiologic techniques such as magnetic resonance imaging. However, the safety of these nanoparticles is still not completely established, particularly to host defense systems that are usually recruited for their clearance from the body. In this paper, given the importance of neutrophils in the immune response of the organism to nanoparticles, the effect of polyacrylic acid (PAA)-coated and non-coated ION on human neutrophils was evaluated *in vitro*, namely their capacity to activate the oxidative burst and to modify their lifespan. The obtained results showed that the studied PAA-coated and non-coated ION triggered neutrophils' oxidative burst in a NADPH oxidase dependent manner, and that PAA-coated ION increased — while non-coated ION prevented — apoptotic signaling and apoptosis. These effects may have important clinical implications in biomedical applications of ION.

1. Introduction

Nanotechnology is nowadays at the forefront in the development of new therapeutic and diagnostic tools in all areas of medicine (Shubayev et al., 2009). A good example of nanotechnology applications is brought by the use of iron oxide nanoparticles (ION), due to their multifunctional properties, conferred by their small size, superparamagnetism, and biocompatibility (Mou et al., 2011). In fact, ION have the potential to be extensively used for the improvement of site-specific drug delivery to cells, tissues, or even organs, as well as in the enhancement of magnetic resonance imaging contrast, hyperthermia treatments in cancer therapy, magnetofection, stem cell therapy and gene delivery (Hong et al., 2011; Muller et al., 2007; Naqvi et al., 2010; Shubayev et al., 2009). To prevent the precipitation of iron oxide cores, ION for medical imaging are always coated with a layer of protective and biocompatible colloid, usually a polymer that acts as a steric and/or electrostatic stabilizer (Roohi et al., 2012). In particular, the polyacrylic acid (PAA) coating is an aqueous soluble polymer with a high density of reactive functional groups that make it very attractive in biomedicine, mainly due to its capability to form flexible polymer chain-protein complexes trough electrostatic, hydrogen bonding or hydrophobic interactions (Pineiro-Redondo et al., 2011).

* Corresponding author. Tel.: +351 220428675.
E-mail address: egracas@ff.up.pt (E. Fernandes).

0378-4274/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2013.11.020

Surface coating and size affect biodistribution, plasma half-life, and extent of cellular uptake of nanoparticles (Muller et al., 2007; Roohi et al., 2012). *In vivo*, large ION (comprised between 60 and 100 nm) are rapidly phagocytosed by cells of the reticuloendothelial system in the liver and spleen, having thereby a short blood half-life. On the other hand, small ION (<60 nm) are not readily phagocytosed, which results in a longer plasma half-life and higher availability to other cells and organs of the immune system (Matsushita et al., 2011; Muller et al., 2007). Although ION may represent extremely useful tools in biomedicine, there are still few studies assessing their possible effects in the above mentioned host defense systems that are usually recruited for their clearance from the body. It was previously reported that ION have the ability to decrease the monocytes' viability (Zhu et al., 2011), as well as to induce the production of reactive oxygen species (ROS) on macrophages and decrease their viability, through apoptosis, in a concentration-dependent manner (Lunov et al., 2010a,b; Naqvi et al., 2010). However, the effect of ION on neutrophils is still to be clarified, this being the purpose of the present study.

Human neutrophils are the most abundant leukocytes in blood and constitute the first line of innate host defense against pathogens and associated acute inflammations (Bockmann et al., 2001; Fadeel et al., 1998; Freitas et al., 2008). Neutrophils are mobilized to the sites of invasion or inflammation, ingesting pathogens into phagosomes (Fadeel et al., 1998; Freitas et al., 2009a,b, 2008). The phagosome fuses with neutrophilic cytoplasmatic granules containing cytotoxic enzymes, namely lysosomal enzymes, as well as NAPDH oxidase and myeloperoxidase, which are responsible for the oxidative burst and consequent generation of ROS (Brasen et al., 2010; Fadeel et al., 1998; Freitas et al., 2009a,b, 2008). While these events are important for the elimination of pathogens, it is not clear how these cells cope with ION, and which consequences ION have on their lifespan.

Neutrophils have a short lifespan that is regulated by the onset of apoptosis. In fact, apoptosis in mature neutrophils is a constitutive process that results in a rapid turnover of the circulating neutrophil population [(5×10^{10} neutrophils per day are released from bone marrow (Goncalves et al., 2010)] with a $t_{1/2}$ of 5 to 6 h *in vivo* and 24 to 36 h *in vitro* (Watson et al., 1998). This process is essential for the normal resolution of inflammation in tissues, because it culminates in the recognition and clearance of the apoptotic neutrophils by macrophages (Rowe et al., 2002). While it has been postulated that these cells undergo apoptosis spontaneously (Goncalves et al., 2010; Rowe et al., 2002), external factors may influence this process, as we have previously shown (Freitas et al., 2013a), and therefore the influence of the different nanoparticles in this process requires further investigation.

Necrosis is an unorganized process associated with extensive damage, resulting in an intense inflammatory response. In neutrophils, this process may occur due to a lack of intracellular adenosine triphosphate (ATP), necessary to apoptosis. Due to the energy-consumptive oxidative burst and consequent depletion of intracellular ATP stores, these cells may be unable to maintain cellular homeostasis and membrane integrity, occurring an influx of water and extracellular ions. This influx will trigger the intracellular organelles and the whole cell swelling, with all the cellular contents being released into the extracellular fluid and surrounding tissues (Kroemer et al., 2007; Turina et al., 2005).

Considering the lack of knowledge on the activation of neutrophils and modulation of their lifespan by ION, the aim of this work was to evaluate the effects of ION in magnetite form (polyacrylic acid (PAA)-coated and non-coated) on human neutrophils, namely their capacity to activate the oxidative burst and to modify their lifespan through necrosis and/or apoptosis.

2. Materials and methods

2.1. Materials

Human venous blood was obtained from healthy human volunteers from Hospital de Santo António (Porto, Portugal). Histopaque 1077, histopaque 1119, Dulbecco's phosphate buffer saline, without calcium chloride and magnesium chloride (PBS) [2.68 mM KCl, 0.14 M NaCl, 1.21 mM KH_2PO_4, 8.10 mM Na_2HPO_4], RPMI 1640 medium, L-glutamine, penicillin, streptomycin, trypan blue solution 0.4%, phorbol 12-myristate 3-acetate (PMA), dihydrorhodamine 123 (DHR), diphenyleneiodonium chloride (DPI), *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide, Ac-Leu-Glu-His-Asp-*p*-nitroanilide, potassium phosphate, phenlymethylsulfonyl fluoride, leupeptin, pepstatin, HEPES and ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma Chemical Co (St Louis, USA). β-Nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), sodium pyruvate, CHAPS, triton™ X-100, dithiothreitol, ferrous chloride, ferric chloride, NH_4OH, KCl and polyacrylic acid PAA (average M_w 1800) were obtained from Sigma-Aldrich (St Louis, USA). Hemacolor® was obtained from Merck (Darmstadt, Germany). (±)-Nutlin-3 was acquired from Cayman (Michigan, USA). Sucrose was obtained from Mallinckrodt Chemical Works (St Louis, USA). Annexin-V-FLUOS Staining Kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Nuclear Extract Kit and TransAM™ p53 Transcription Factor Assay Kits were acquired from Active Motif (La Hulpe, Belgium).

2.2. Methods

2.2.1. Synthesis of iron oxide nanoparticles

ION "non-coated" magnetite particles were prepared following previous well-known procedures with some modifications (Massart et al., 1995). In brief, the procedure is based on the chemical co-precipitation of a mixture of Fe(II) and Fe(III) chloride salts (molar ratio 2:1) using NH_4OH in a degassed 1 M KCl aqueous solution at 60 °C. The dark precipitate was washed several times with deoxygenated water, and finally the particles were stored at pH 9.6 (well-above their isoelectric point: 6.5). For the PAA-coated particles, PAA (25% w/w with respect to the Fe(II) salt) was added to the reaction medium.

2.2.2. Characterization of iron oxide nanoparticles

Non-coated and PAA-coated ION were characterized using transmission electron microscopy (TEM) (Hitachi H-7000, Japan). Determination of the hydrodynamic size and zeta potential of the nanoparticles in water suspensions, in function of pH and [NaCl], as well as in the medium used for the studies on human neutrophils [RPMI 1640 (pH = 7.4) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin] were made using a nanoparticle analyzer SZ-100 (HORIBA Scientific) (DPSS laser 532 nm). Before the dilutions, ION were sonicated for 5 min in order to avoid the formation of aggregates before the preparation of the samples.

2.2.3. Isolation of human neutrophils by the gradient density centrifugation method

Following informed consent, venous blood was collected from healthy human volunteers by antecubital venipuncture, into vacuum tubes with K_3EDTA. The isolation of human neutrophils was performed by the gradient density centrifugation method as previously reported in (Freitas et al., 2008). RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin was the incubation medium used.