Colloids and Surfaces B: Biointerfaces 134 (2015) 122–130

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

In vitro hematological and *in vivo* immunotoxicity assessment of dextran stabilized iron oxide nanoparticles

Sheeja Liza Easo, Mohanan P.V.*

Division of Toxicology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojapura, Thiruvananthapuram 695 012, Kerala, India

ARTICLE INFO

Article history:
Received 5 April 2015
Received in revised form 18 June 2015
Accepted 22 June 2015
Available online 29 June 2015

Keywords:
Iron oxide nanoparticles
Hemolysis
Platelet aggregation
Lymphocyte proliferation
Cytokines
Intravenous

ABSTRACT

Iron oxide nanoparticles have attracted enormous interest as potential therapeutic agents. The purpose of this study was to examine the *in vitro* hematological toxicity and *in vivo* immune response toward previously synthesized and characterized dextran stabilized iron oxide nanoparticles (DIONPs) developed for hyperthermia application. Peripheral whole blood from human volunteers was used to investigate hemolysis, platelet aggregation, lymphocyte proliferation and cytokine mRNA expression induced by DIONPs *in vitro*. In the concentration range of 0.008–1 mg/ml, DIONPs did not induce relevant levels of hemolysis or platelet aggregation. Assessment of lymphocyte function showed significant suppression of the proliferation activity of T-lymphocytes in cultures stimulated with the mitogen phytohemagglutinin (PHA). In addition, inhibition of PHA-induced cytokine mRNA expressions was also seen. However, systemic administration of DIONPs resulted in enhanced proliferation of mitogen-stimulated spleen derived lymphocytes and secretion of IL-1β at day 7 post exposure. In conclusion, our results demonstrate that immune response is influenced variably by nanoparticles and its degradation milieu. Further investigation of the observed immunosuppressive effects of DIONPs in immune stimulated animal models is required to assess the functional impact of such a response.

1. Introduction

Development of nanoparticles for various biomedical applications has greatly increased. This is reflected in the variety of commercialized nanotechnology based products, as well as those in clinical testing for therapeutic and diagnostic purposes. Undoubtedly, the aim in developing nanoparticle based products is to bring considerable benefits to the intended field of application. The potential of nanoparticles to interact with cellular systems owing to its small size and enhanced surface area is widely acknowledged. While such interactions may be beneficial if controlled suitably; conversely, they could result in undesirable toxicity. Intravenous administration of nanoparticles is sure to allow their interaction with blood components, prior to subsequent distribution to tissues and cells.

Preclinical testing of nanoparticles is an essential requirement for any kind of *in vivo* application [1,2]. The surface properties of nanoparticles can greatly affect their compatibility in the bloodstream. On the other hand, blood constituents can react immunologically to render nanoparticles and their drug complexes inactive [3]. RBCs and the multi-component hemostatic cascades, which include platelets and plasma proteins, are crucial in maintaining homeostasis and fluidity of blood in circulation. Hemolytic potential and platelet reactivity has been demonstrated for several nanoparticle preparations [4–7]. Existing reports clearly suggest that nanoparticles interact with immune cells and may cause their stimulation or suppression [8–10]. The ability of nanoparticles to induce an immune response is, in turn, determined by particle size, charge and hydrophobicity. Additionally, it is influenced by several other factors, including the surface targeting moieties, the therapeutic payload, the animal model and the route of administration [11]. Consequently, examination of the hemolytic potential, the platelet aggregation capacity as well as the immune response is crucial to evaluating the toxicity of nanoparticles.

Iron oxide nanoparticles hold immense promise as diagnostic and therapeutic agents in oncology. Their intrinsic physical properties make iron oxide nanoparticles particularly interesting for simultaneous drug delivery, molecular imaging and localized hyperthermia applications. It is well known that iron has immunoregulatory properties, and any alterations in cellular iron levels may affect immune response [12] and inflammatory signaling [13]. Our long-term objective is to use iron oxide nanoparticles as ther-

* Corresponding author.
E-mail address: mohanpv10@gmail.com (P.V. Mohanan).

http://dx.doi.org/10.1016/j.colsurfb.2015.06.046
0927-7765/

apeutic agents for hyperthermia treatment; and hence, we have developed dextran stabilized iron oxide nanoparticles (DIONPs) for this purpose. Since the physicochemical properties of nanoparticles are relevant in toxicology and affect their functionality, we had previously performed a complete characterization of DIONPs [14]. In the context of their biomedical application, we consider it crucial to comprehend the outcome of the interactions of DIONPs with blood components that would most certainly come into contact with nanoparticles immediately after systemic absorption. Thus, in this study we report on the interaction of DIONPs with RBCs, platelets and its immunogenic effect on human primary lymphocytes in terms of immunostimulation and immunosuppression, as well as the cytokine response. These investigations are expected to reveal some important elements addressing the toxicity of DIONPs from a preliminary safety standpoint. In addition, we have addressed the delayed immune response in spleen when DIONPs were administered intravenously in rats for a period of 14 days.

2. Materials and methods

2.1. Materials

Instruments used include dynamic light-scattering (DLS) and zeta-potential analyzer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK), transmission electron microscope (TEM) (Hitachi H-7650, Japan), fourier transform infrared spectrometer (Nicolet 5700 FTIR, Thermo Electron Corporation, US), microplate reader (EL_x808, BioTek, US), automatic hematology analyzer (SYSMEX K4500, Japan), CO_2 incubator (MCO-18AIC, Sanyo, Japan), Biophotometer (Eppendorf AG, Hamburg, Germany), iQ5 thermal cycler (Bio-Rad Labortaries, USA) scintillation counter (Triathler, Hidex, Turku, Finland). Chemicals include RPMI-1640 medium, rat tail collagen, lipopolysaccahride (LPS), phytohemagglutinin (PHA), polyethylene glycol (PEG) (Sigma), Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen), cyanmethemoglobin (CMH) reagent (Randox Laboratories Ltd., UK), hemoglobin standard (Span Diagnostics Ltd., India), tritiated thymidine [3H] (American Radiolabeled Chemicals, Inc.), Triton X-100 (S D Fine-Chemicals, India), sterile saline (Parenteral Drugs Ltd., India), TRI reagent (Ambion), Quantitative PCR (qPCR) kit (Eurogentec).

2.2. Nanoparticles

DIONPs were synthesized and characterized as described previously [14]. Particle core size and morphology were examined using TEM at an accelerating voltage of 80 kV. A drop of the nanoparticle dispersion was deposited on formvar coated grids and air-dried for several hours before analysis. The hydrodynamic radius and polydispersity index (PdI) of nanoparticles was determined using DLS. Samples taken from concentrated nanoparticle (~35 mg/ml) dispersion was diluted in water and complete culture medium to achieve a concentration of 0.05 mg/ml and gently stirred to ensure homogeneity. Appropriate amount of the diluted samples was transferred to disposable cuvettes for analysis. DLS measurements were carried out at 25 °C, at a scattering angle of 173°. The Z-average intensity-weighted radii were calculated using the instrument software. Zeta potentials of freshly prepared 0.05 mg/ml nanoparticle dispersions in water and complete culture medium were also measured at 25 °C. All measurements were calculated as an average of three runs containing 12 measurements per run after 4 h incubation. To evaluate dextran coating on nanoparticle surface and the nature of its bonding, FTIR analysis was performed. Briefly, freeze dried nanoparticle preparations were mixed with potassium bromide to form a fine powder. The powder was compressed into a thin pellet, and the FTIR spectrum was recorded between 4000 cm^{-1} and 400 cm^{-1} at a resolution of 4 cm^{-1}.

Nanoparticle concentration was expressed as mg of Fe per ml in all experiments. For cell exposure experiments, DIONPs dispersion in varying concentrations ranging from 0.008–1 mg/ml was freshly prepared by diluting the DIONPs suspension in the appropriate medium.

2.3. In vitro *studies*

2.3.1. Subjects

A total of six volunteers participated in this study (male and female). Blood was collected by venipuncture into tubes containing appropriate anticoagulants. Blood samples obtained from participants were pooled for all experiments. Study participants signed an informed consent form, and the study was approved by the Institutional Ethics Committee (ECR/189/Inst/KL/2013).

2.4. Hemolysis assessment

Hemolysis assay was performed as described in http://ncl.cancer.gov/NCL_Method_ITA-1.pdf. Briefly, blood samples were collected into vials with heparin as the anticoagulant. The total hemoglobin concentration of heparinized human whole blood was measured using cyanmethemoglobin method based on a hemoglobin standard curve at an absorbance wavelength of 540 nm. Blood was diluted to obtain a final hemoglobin concentration of 10 mg/ml with Ca^{2+}/Mg^{2+} free DPBS. Nanoparticle samples at four different concentrations (0.008. 0.04, 0.2 and 1 mg/ml) along with positive (Triton X-100) and negative controls (PEG) were analyzed. Aliquots (100 μl) of the nanoparticle suspension were added to microcentrifuge tubes, followed by the addition of 700 μl of Ca^{2+}/Mg^{2+} free DPBS. To each of these tubes, 100 μl of diluted blood was added. Tubes were incubated in a 37 °C water bath for 3 h, with gentle inversion of the sample tubes every 30 min. Following incubation, tubes were centrifuged at 800× *g* for 15 min at room temperature. The supernatant was mixed in a 1:1 ratio with CMH reagent and analyzed at 540 nm in a microplate reader. Sample absorbance was corrected for background interference (particles in DPBS without blood). The concentration of cell-free hemoglobin in each sample was assessed from the hemoglobin standard curve by accounting for the 16-fold dilution factor for samples and controls. Finally, percentage hemolysis was obtained by dividing each sample's cell-free hemoglobin concentration by total hemoglobin concentration (10 mg/ml).

2.5. Evaluation of platelet aggregation

To study the effect of nanoparticles on platelet aggregation, whole human blood collected in vials containing sodium citrate anticoagulant was centrifuged for 8 min at 200× *g* to obtain platelet rich plasma (PRP). This PRP was treated separately with DIONPs (0.008, 0.04, 0.02 and 1.0 mg/ml), PBS (negative control) and collagen (positive control) for 15 min at 37 °C. To investigate whether DIONPs could interfere with collagen-induced platelet aggregation, PRP was also treated with a mixture of collagen and DIONPs under the same conditions. A single-platelet count was then conducted using an automatic hematology analyzer. A decrease in single platelet count due to platelet aggregation was used to calculate percentage aggregation. A detailed protocol is available at http://ncl.cancer.gov/NCL_Method_ITA-2.pdfhttp://ncl.cancer.gov/NCL_Method_ITA-2.pdf