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In vivo uptake and acute immune response to orally administered chitosan and PEG coated PLGA nanoparticles

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ABSTRACT

Nanoparticulate drug delivery systems offer great promise in addressing challenges of drug toxicity, poor bioavailability and non-specificity for a number of drugs. Much progress has been reported for nano drug delivery systems for intravenous administration, however very little is known about the effects of orally administered nanoparticles. Furthermore, the development of nanoparticulate systems necessitates a thorough understanding of the biological response post exposure. This study aimed to elucidate the *in vivo* uptake of chitosan and polyethylene glycol (PEG) coated Poly, DL, lactic-*co*-glycolic Acid (PLGA) nanoparticles and the immunological response within 24 h of oral and peritoneal administration. These PLGA nanoparticles were administered orally and peritoneally to female Balb/C mice, they were taken up by macrophages of the peritoneum. When these particles were fluorescently labelled, intracellular localisation was observed. The expression of pro-inflammatory cytokines IL-2, IL-6, IL-12p70 and TNF-α in plasma and peritoneal lavage was found to remain at low concentration in PLGA nanoparticles treated mice as well as ZnO nanoparticles during the 24 hour period. However, these were significantly increased in lipopolysaccharide (LPS) treated mice. Of these pro-inflammatory cytokines, IL-6 and IL-12p70 were produced at the highest concentration in the positive control group. The anti-inflammatory cytokines IL-10 and chemokines INF-γ, IL-4, IL-5 remained at normal levels in PLGA treated mice. IL-10 and INF-γ were significantly increased in LPS treated mice. MCP-1 was found to be significantly produced in all groups in the first hours, except the saline treated mice. These results provide the first report to detail the induction of cytokine production by PLGA nanoparticles engineered for oral applications.

Introduction

Nanoparticles have to date been extensively used for various applications including drug delivery (Liversidge and Cundy, 1995; Duncan, 2005), tissue engineering (Langer, 2000) and imaging (Bruchez, 2005). Their physiochemical properties including their small size and large surface area have led to these advances. In drug delivery, they have been reported to significantly improve the bioavailability of drugs and minimise drug toxicity (Bawarski et al., 2008; Farokhzad and Langer, 2006; Langer, 2000), thus leading to more efficient therapies.

In drug delivery, the nano size range of particles is the 'holy grail' of efficient drug delivery, facilitating efficient uptake of the drugs via various uptake mechanisms (Jones et al., 2003). Intracellular uptake of the drugs is not very efficient with conventional formulations, albeit its necessity, primarily for drugs against intracellular microorganism. This shortfall is addressed by nanoparticulate drug delivery systems, where increased intracellular concentrations of drugs are observed when the drugs were nanoencapsulated (Kisich et al., 2007). The first cellular targets for nanoparticles are macrophages and dendritic cells (DC), which are professional antigen presenting cells that are at the fore front of the body's defence system. After engulfing foreign material, they mature to become active antigen presenting cells expressing specific maturation markers such as CD11c and MOMA-2 and others (Noti and Reinemann, 1995). In addition, when these cells are activated, they produce cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-10, IL-18 and tumor necrosis factor alpha (TNF-α) and chemokines that attract other inflammatory cells to the site of inflammation (Anderson et al., 2008).

Since nanoparticles are foreign, their uptake may result in the release of the pro-inflammatory cytokines (Chang, 2010; Lee et al., 2009). The immunogenicity of synthetic polymers highly depended

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on their size, shape, composition, surfactant properties, electrical charge and on the inherent ability of the host to recognise them. Furthermore, the oxidative potential of nanoparticles is another important parameter for evaluating their inflammatory or immunological responses. Synthetic polymers used in biological applications, such a drug delivery and tissue engineering, must therefore be biocompatible and biodegradable, i.e. their introduction into the body must not provoke a hazardous reaction (Kim et al., 2007; Rihova, 2002). Various groups are thus proposing studies that will measure the cell viability, inflammatory effects and biomedical effects of nanomaterials (Kim et al., 2007).

In this study we investigated the *in vivo* uptake of chitosan and polyethylene glycol (PEG) coated PLGA (referred to in this manuscript as PLGA nanoparticles) nanoparticles post oral administration. These particles are currently being explored for delivery of various compounds including antibiotics for the treatment of tuberculosis (TB). Furthermore, we analysed the *in vivo* immunological response to the uptake of these particles. This is the first study to analyse the uptake of PLGA nanoparticles *in vivo* and in conjunction evaluate the subsequent immune reaction by analysing the concentration profile of the secreted pro- and anti-inflammatory cytokines.

Materials and methods

Preparation of PLGA particles

Poly, DL, lactic-*co*-glycolic Acid (PLGA) 50:50 (Mw: 45,000–75,000), nanoparticles were prepared using a modified double emulsion solvent evaporation technique (Lamprecht et al., 1999). An aqueous phosphate buffer solution (PBS) pH 7.4 was emulsified for a short period with a solution of 100 mg PLGA dissolved in 8 ml of ethyl acetate (EA), by means of a high speed homogeniser (Silverson L4R) with a speed varying between 3000 and 5000 rpm. This water-in-oil (w/o) emulsion obtained was transferred into a specific volume of an aqueous solution of 1% w/v of the polyvinyl alcohol (PVA) (Mw: 13,000–23,000, partially hydrolysed (87–89%)) as a stabiliser. The mixture was further emulsified for 5 min by homogenisation at 5000 or 8000 rpm. These methods were carried out aseptically using a laminar airflow chamber. The double emulsion (w/o/w) obtained was directly fed into a bench top Buchi mini-spray dryer (Model B-290) and spray dried at a temperature ranging between 95 and 110 degrees Celsius (°C), with an atomizing pressure varying between 6 and 7 bars.

1% PEG was used in the formulation as an excipient to increase the *in vivo* residence time of nanoparticles in the blood stream (Torchilin and Trubetskoy, 1995). In order to enhance the uptake in the gastrointestinal tract, a mucoadhesive and positively charged ligand, chitosan was added in the formulation as recommended in previous reports (Cui et al., 2006; Takeuchi et al., 2005). 3% (volume/volume) chitosan was added to the formulation. Rhodamine 6G (Sigma, South Africa) labelled PLGA nanoparticles were prepared using the same method, where Rhodamine 6G was added in the aqueous phase of the emulsion.

Particle characterisation

Particle size, zeta potential and composition. Particle size and size distribution of PLGA and ZnO particles as well as polystyrene beads were measured by Dynamic Laser Scattering (DLS) or Photon Correlation Spectroscopy (PCS) using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., UK). For each sample 1–3 mg of nanoparticles were suspended in filtered water (0.2 μm filter), then vortexed and/or sonicated for a few minutes. Each sample was measured in triplicate. The zeta potential was also determined using the same instrument. Surface morphology of PLGA nanoparticles was studied by scanning electron microscopy (LEO 1525 Field Emission SEM). The chitosan content in the PLGA particles was characterised via Fourier Transformed Infrared (FT-IR) using the PerkinElmer Spectrum 100 FT-IR Spectrometer.

Test for pyrogens in the particles. The PryoDetect System supplied by Biotest AG (Germany) was used for the analysis of pyrogen content in the PLGA, polystyrene and ZnO nanoparticles, according to the manufactures' instructions. Briefly, the particles were mixed with sterile cryo blood (provided with the kit) in a cell culture plate in triplicate and kept in a CO_2 incubator at 37 °C for 24 h. The test detects for IL-1B produced by blood monocytes in the presence of pyrogens. For the detection of IL-1B, the nanoparticle–blood mixture was transferred into an ELISA microplate coated with antibody specific for IL-1B and incubated for 2 h, then washed. IL-1B molecules present in the supernatant would then bind to the immobilised antibody. A horseradish peroxidase (HRP) labelled anti-human polyclonal antibody specific for IL-1B was added and incubated for 1 h and thereafter washed. A substrate provided with the kit was added and incubated at room temperature for 20 min resulting in a colour reaction and a stop solution added thereafter. The plate was then analysed at 450 nm on the BIO-TEK ELx800 plate reader. The standard curve was generated using a different concentration of the endotoxin standard provided with the kit. The data was analysed using the Combistats software programme and presented in Endotoxin Units per ml (EU/ml).

Animals. Unchallenged, healthy Balb/C male mice weighing 20–25 g were selected and housed under standard environment conditions at ambient temperature of 25 °C, and supplied with food and water *ad libitum*. Ethics approval was obtained from this study from the MRC Ethics Committee for Research on Animals (ECRA), Tygerberg, Cape Town, South Africa.

In vivo particle uptake. To evaluate particle uptake, saline was administered via the oral and intraperitoneal (i.p) routes respectively to mice as a negative control (Group 1) and 4% Brewers thioglycolate broth as a positive control (Group 2). A volume of 0.2 ml of 20 mg/ml Rhodamine 6G labelled nanoparticles was administered via the oral route once daily over five days (Group 3) and another group via the intraperitoneal route once only over the period of five days (Group 4). PLGA nanoparticles that were not fluorescently labelled were also administered at the same concentration to another group in a similar manner (Group 5).

This specific dose of PLGA was selected as it corresponds to the concentration of PLGA particles used in our research group for the administration of PLGA encapsulated anti-TB drugs, at a drug dose

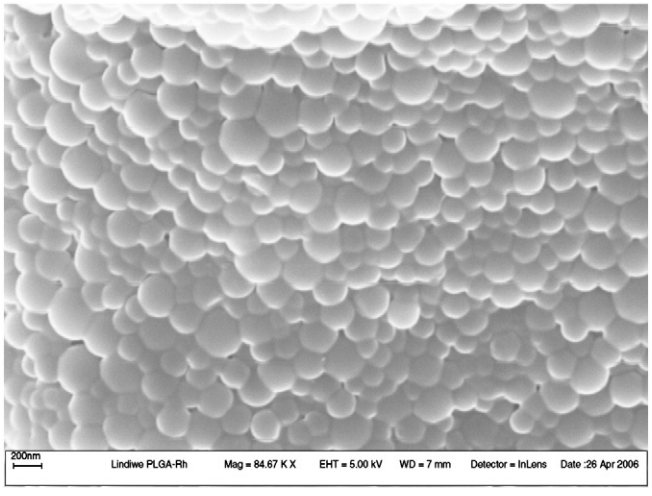

Fig. 1. SEM image of Rhodamine labelled PLGA nanoparticles.