Pharmacokinetics and bio-distribution of novel super paramagnetic iron oxide nanoparticles (SPIONs) in the anaesthetized pig

Deirdre Edge,††‡‡ Christine M Shortt,§†† Oliviero L Gobbo,† Stephanie Teughels,‡
Adriele Prina-Mello,§§ Yuri Volkov,§§ Peter MacEneaney,** Marek W Radomski†† and Farouk Markos*†

*Department of Physiology, University College Cork, Cork, †School of Pharmacy and Pharmaceutical Sciences and Trinity Biomedical Sciences Institute, Dublin, Ireland, ‡PEPRIC nv, Leuven, Belgium, §School of Medicine, ‡‡CRANN, Trinity College Dublin, **Mercy University Hospital, and ‡‡‡Department of Physiology, School of Medicine, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland

SUMMARY

Manufactured nanomaterials have a variety of medical applications, including diagnosis and targeted treatment of cancer. A series of experiments were conducted to determine the pharmacokinetic, biodistribution and biocompatibility of two novel magnetic nanoparticles (MNPs) in the anaesthetized pig. Dimercaptosuccinic acid (DMSA) coated super-paramagnetic iron oxide nanoparticles (MF66-labelled 12 nm, core nominal diameter and OD15 15 nm); at 0.5, or 2.0 mg/kg were injected intravenously. Particles induced a dose-dependent decrease in blood pressure following administration which recovered to control levels several minutes after injection. Blood samples were collected for a 5-h period and stored for determination of particle concentration using particle electron paramagnetic resonance (pEPR). Organs were harvested post-mortem for magnetic resonance imaging (MRI at 1.5 T field strength) and histology. OD15 (2.0 mg/kg) MNP had a plasma half-life of approximately 15 min. Both doses of the MF66 (0.5 and 2.0 mg/kg) MNP were below detection limits. MNP accumulation was observed primarily in the liver and spleen with MRI scans which was confirmed by histology. MRI also showed that both MNPs were present in the lungs. The results show that further modifications may be required to improve the biocompatibility of these particles for use as diagnostic and therapeutic agents.

Key words: biodistribution, blood pressure, electron paramagnetic resonance (pEPR), magnetic nanoparticles, magnetic resonance imaging (MRI).

INTRODUCTION

Cancer is a leading cause of death worldwide with 8.2 million cancer-related deaths recorded in 2012.¹ The most common treatments for cancer include surgery, chemotherapy and radiology; these treatments often result in intense side effects for the patient and in some cases can lead to discontinuation of therapy prior to tumour eradication.² Significant advances have been made in cancer therapy, although selective elimination of cancer cells remains elusive. Therefore, it is vital that novel methods for the early detection and selective treatment of cancer are developed to aid and enhance conventional methods.

MULTIFUN is a large European project focused on the development and validation of new systems based upon minimally invasive nanotechnology for the treatment of breast and pancreatic cancer. The project aims to deliver a multimodal approach for the early detection and treatment of cancer using iron oxide magnetic nanoparticles (MNPs) for targeted drug delivery and diagnostic imaging is a novel and exciting new concept. MNPs have been used in an array of applications, from contrast agents for magnetic resonance imaging (MRI) to magnetic carriers for enhancing drug delivery.³

MNPs that can act as both a diagnostic tool and as an agent for targeted-drug delivery are clinically important. Superparamagnetic iron oxide nanoparticles (SPIONs) have an iron oxide core and therefore can be detected using MRI, and are also useful as heating targets for magnetic hyperthermia.⁴

It has been shown that SPION MNPs are relatively stable in vivo in a small animal study by Inversen and co-workers.⁵ The properties of the particles (e.g., composition, size, shape and surface coating) will affect particle biodistribution and biocompatibility.⁶,⁷ However, the determination of biodistribution and biocompatibility of these particles is essential in a larger animal model prior to clinical application. Therefore, the aim of this study was to examine the effects of two lead-candidate MULTIFUN nanoparticles, MF66 (12 nm diameter) and OD15 (15 nm diameter), on basic cardiovascular parameters such as blood pressure, following injection and hence to assess their pharmacokinetic and biodistribution patterns in a large animal model in vivo. Both particles were found to be non-toxic at very high dosages in vitro and in small animal in vivo (mice).
studies carried out by colleagues in our consortium (unpublished observations).

RESULTS

Blood pressure response

MNPs were injected intravenously (i.v.) via the femoral vein. A significant drop in mean arterial blood pressure (MAP) was observed following 0.5 mg/kg of MF66 (Fig. 1c, 96 ± 3 vs 64 ± 8 mmHg, \( P = 0.015; n = 5; \) Student’s paired \( t \)-test) and 2.0 mg/kg of MF66 (Fig. 1e, 106 ± 5 vs 44 ± 6 mmHg, \( P = 0.0003; n = 4; \) Student’s paired \( t \)-test). In addition, 0.5 mg/kg of OD15 (Fig. 1d, 103 ± 4 vs 67 ± 13 mmHg, \( P = 0.03; n = 5; \) Student’s paired \( t \)-test) and 2.0 mg/kg of OD15 (Fig. 1f, 96 ± 7 vs 47 ± 5 mmHg, \( P = 0.0002; n = 4; \) Student’s paired \( t \)-test) caused a significant transient drop in blood pressure. Blood pressure returned to baseline levels within minutes of the injection.

Particle concentration in blood

Blood samples were taken from the animals at specific time points following MF66 and OD15 injection. MNP concentration was determined using pEPR. The levels of MF66 in blood were below pEPR detection limits of 0.9 mg/L (Fig. 2a). In contrast, blood levels were transiently elevated following injection of 2.0 mg/kg, but not 0.5 mg/kg OD15 (Fig. 2b). No particles could...
be detected in the blood approximately 30 min post-injection of 2.0 mg/kg OD15 (Fig. 2b).

Particle concentration in lung tissue

MNP concentration was determined in lung tissue using the same pEPR technique. MF66 and OD15 (2.0 mg/kg, \( n = 3 \); 0.5 mg/kg, \( n = 3 \)) caused a dose-dependent accumulation of iron particles in lung tissue compared to control animals (Fig. 3a,b; \( P < 0.05 \), ANOVA).

MRI

Qualitative T2* MRI images were taken post-mortem from control animals, OD15-treated animals (2.0 mg/kg and 0.5 mg/kg) and MF66- treated animals (2.0 mg/kg and 0.5 mg/kg). Both OD15 and MF66 MNPs accumulated in the liver (Figs 4,5) and the spleen (not shown) in a dose dependent manner. In contrast, there was negligible particle accumulation observed in the kidneys and the heart (Fig. 5). The precise localization of MNPs in the lung is uncertain as MRI could not differentiate between parenchymal and air-containing parts of this organ (data not shown). Interestingly, we observed a greater aggregation of MF66 MNP compared to OD15 MNP in the liver suggesting that OD15 was more evenly distributed in this organ.

Histology

Representative images of Prussian blue staining in lung tissue from control (Fig. 6a,d), MF66-treated (Fig. 6b,e) and OD15-treated (Fig. 6c,f) animals are shown at both low and high magnification for qualitative accumulation measurement. Following MF66 (2.0 mg/kg) treatment, MNPs (identified by blue dots) are observed in the high magnification image (Fig. 6e). Similarly, animals treated with OD15 (2.0 mg/kg) also present with MNPs in their lung tissue (Fig. 6f).

DISCUSSION

The use of iron oxide nanoparticles as potential tool for the diagnosis and elimination of cancer offers great benefits to the field of nanomedicine and cancer therapy. With this in mind, it is vital that the properties of these MNPs are examined prior to clinical application. This study examined the pharmacokinetics and biodistribution of two iron oxide MNPs in a large animal. Swine were selected as the experimental model due to its similar anatomical and physiological characteristics with humans.

The main findings of the present study are: (i) MNPs (MF66 and OD15) induce a significant transient decrease in blood pressure; (ii) OD15 (2.0 mg/kg) was cleared from the circulation 30 min post injection; (iii) MNPs (MF66 and OD15) are...
localised mainly to the liver, spleen and lungs following administration.

**Blood pressure**

Little work has been conducted to date on the cardiovascular effects of MNP’s following i.v. administration, especially in a large animal. A significant transient decrease in MAP following administration of both aqueous-based method (MF66) and organic-based (OD15) MNP was observed. In a conscious rat model, MAP was reportedly significantly decreased following administration of an array of drug-free bare nanoparticles ranging in size from 90 to 163 nm at a dose of 5.0 mg/kg,\(^{11}\) as well as following injection of polyacrylic acid (PAA) coated iron oxide MNPs at a dose of 10.0 mg/kg in a conscious mouse model.\(^{5}\)

The particles investigated in the current study, were much smaller in size, 12.0 and 15.0 nm compared to those tested by Vlasova et al.,\(^{11}\) suggesting this drop in MAP is not solely dependent on the size of the particles. The decrease in MAP following both doses of MNPs (0.5 and 2.0 mg/kg) in pigs, is smaller than those observed following 5.0 and 10.0 mg/kg in rats and mice, respectively.\(^5,^{11}\)

Vlasova et al.\(^{11}\) proposed the decrease in MAP was due to both endothelium-dependent and independent arterial vasodilation. In a pilot study in rats (unpublished observation), arterial dilatation was assessed by measuring renal blood flow following injection of 2.0 mg/kg of both MF66 and OD15. No significant effect on renal blood flow was observed, which suggests that the particles do not cause a global arterial dilatation. This current pig study was acute, and the decrease in MAP was almost immediate, within 2 min of the MNPs being flushed into the system, similar to the study carried out by Vlasova et al.\(^{11}\) who observed the decreased MAP 6, 15 min post intravenous injection of some of the MNPs. Contrastingly, the fall in MAP in mice was more latent, with the effects on MAP taking place 12–24 h post injection.\(^5\) This group attributed the effects to decreased contractility of the mesenteric arteries, which may be another potential mechanism in the pig; however immediate dilator effects are most probable. Elucidating the effects of MNPs on cardiovascular function in disease states is of importance for future studies, as
many potential patients receiving MNPs are most likely have co-

Pharmacokinetics

Determination of the circulating levels of the iron oxide nanoparticles is important in gaining an insight into their pharmacokinetic behaviour. Although administration of MF66 (0.5 and 2.0 mg/kg) and OD15 (0.5 mg/kg) did not result in significant increases in circulating MNP levels, larger doses could potentially be toxic taking into account that the recommended clinical dose is 0.56 mg Fe/kg of some approved SPIONs.13 Therefore, higher doses were not explored in this study. Using the pEPR method we established that OD15 (2.0 mg/kg) is eliminated from the circulation in 30 min, which is similar to results obtained with DMSA coated particles in a rat model,13 who reported a circulation half-life of 10 min. Nanoparticles were administered as a single intravenous bolus allowing for maximum bioavailability. The short circulation time of the SPIONs has been long noted as an issue,14 especially for biomedical applications, a MNP elimination time of 30 min may be sufficient for some imaging applications such as MRI; however, it is unlikely to be long enough for anticancer drug delivery systems.

Many techniques cannot distinguish between exogenous and endogenous iron (i.e. iron bound to haemoglobin), for example inductively coupled plasma mass spectroscopy (ICP-MS).14,15 Although ICPMS is very sensitive, endogenous iron can affect the reading, especially when biological samples are assessed.

Moreover, piglets are born with limited iron stores, and so they are given a subcutaneous injection of iron-dextran soon after birth.16 The particle electron paramagnetic resonance (pEPR) technique is based on electron paramagnetic resonance at low magnetic fields, and is selective for the magnetisation spin of MNPs, thereby allowing us to distinguish between endogenous and exogenous iron.17

Biodistribution

Biodistribution of MNPs likely depends on key properties such as their size, shape, and surface characteristics.6 Many studies in animal models have detailed accumulation of iron oxide preferentially in the liver and spleen.6,18–20 Jain et al.18 reported around 55% of the injected dose of iron was localized in the liver 6 h post injection. Qualitative MRI indicated that the majority of the injected particles for both MF66 and OD15 at both doses of 0.5 and 2.0 mg/kg (with a stronger darkening of the liver and spleen parenchyma at 2 mg/kg) were localised in the liver 5 h post-injection. The rapid clearance of MNPs by the reticular endothelial system (RES) is an obstacle for efficient biomedical applications. It is no surprise that MNPs are taken up by the RES due to the high level of vascularisation and permeability,20 as well as macrophage content. The process of opsonisation has been previously reported whereby MNPs are rapidly cleared from the circulation by macrophages of the liver.21,22 The lungs are also a highly vascularized and monocyte rich organ, MNPs accumulation has also been reported by many in the lungs.13

Fig. 6 Prussian blue staining in lung tissue. Representative images of lung tissue from (a) control, (b) MF66-treated and (c) OD15-treated animal taken at low magnification (×10). At higher magnification (×40) it is clear that both (e) MF66-treated and (f) OD15-treated stain positive for iron particles (blue spots) compared to (d) control lung tissue.
pEPR analysis we observed a dose dependent accumulation of both MF66 and OD15 in pig lungs compared to controls, 5 h post administration. This was confirmed by histological staining with Prussian blue, showing MNPs localised in alveolar tissue. Other researchers have reported lung accumulation as soon as 30 min post administration,\(^{23,24}\) with associated inflammation.\(^{23}\) The presence of MNPs in the lungs is a concern, although MNP accumulation in mouse lungs reportedly decreases over time, 30 min – 90 days, without associated toxicity.\(^{23}\) The present study was acute, with one final time point of 5 h, a temporal study in the large animal would be of benefit in establishing the consequences of longer exposure in a large animal model.

In conclusion, DMSA coated superparamagnetic iron oxide nanoparticles induce a transient yet significant decrease in MAP. Particles remained in the circulation for up to 30 min and were accumulated in the liver, spleen and lungs. Whether this suggests that DMSA polymeric surface coating could be potentially exploited for further cancer-targeted moiety functionalization or else chemically modified to avoid immunoresponse activity \(in\) \(vivo\) requires further study. Furthermore, other surface coating \((s)\), for example polyethylene glycerol \((\text{PEG})\), may be beneficial for future studies as PEG, a polymeric coating has been shown to delay macrophages particles phagocytosis,\(^{12}\) potentially prolonging the circulation time of MNPs.

**METHODS**

**Ethical approval**

This investigation was carried out under licenses issued by the Department of Health Ireland as directed by the Cruelty to Animals Act Ireland and European Union and International Statutory Instructions.

**Nanoparticle synthesis**

The biodistribution of two dimercaptosuccinic acid \((\text{DMSA})\) coated magnetite \((\text{Fe}_3\text{O}_4)\) nanoparticles, MF66 \((12 \text{ nm nominal core diameter})\) and OD15 \((15 \text{ nm nominal core diameter})\) nanoparticles, were investigated. MF66 nanoparticles were produced in aqueous solvents \((\text{Liquid Research, Bangor, Wales})\) and OD15 \((\text{CSIC, Madrid, Spain})\) was synthesised via an organic route, as previously described.\(^8,9\)

**Surgery and instrumentation**

Twenty-one female landrace pigs \((20–25 \text{ kg})\) were sedated with ketamine \((14.0 \text{ mg/kg})\) and xylazine \((2.7 \text{ mg/kg})\) intra muscularly \((\text{i.m.})\). A cannula was inserted into an ear vein and the animal was anaesthetized with a bolus, followed by a continuous infusion of sodium pentobarbital \((\text{induction 30.0 mg/kg; maintenance 6 mg/kg/h i.v.})\). The continuous infusion was maintained via a catheter inserted into the jugular vein, using an infusion pump \((\text{Harvard apparatus, Holliston, MA, USA})\). End-tidal carbon dioxide \((\text{ETCO}_2)\), arterial oxygen saturation \((\text{S}_\text{aO}_2)\), core body temperature and electrocardiography \((\text{ECG})\) were continuously monitored with a SurgiVet AdvisorVital Signs Monitor \((\text{Smiths Medical, Dublin, Ireland})\). Arterial \(\text{pH, PCO}_2\) and \(\text{PO}_2\) were assessed using a hand held i-STAT blood gas analyser \((\text{Abbot Point of Care Inc, Princeton, NJ, USA})\) and maintained within their normal ranges.

Following tracheotomy, animals were ventilated with 40% \(\text{O}_2\) in room air using a Harvard ventilation pump at a rate adjusted to keep end-tidal and arterial \(\text{PCO}_2\) within a normal range. A cannula attached to a pressure transducer \((\text{Grass; Grass Technologies, West Warwick, RI, USA})\) was inserted into the left carotid artery for measurement of arterial blood pressure. A cannula was also inserted into the femoral vein for nanoparticle injection and venous blood sampling throughout the day.

**Protocol**

A known dose of dimercaptosuccinic acid \((\text{DMSA})\) coated nanoparticles \((\text{MF66 and OD15})\) was prepared \((0.5, \text{ or } 2.0 \text{ mg/kg})\) for venous injection. An equivalent volume of saline was prepared and injected into the femoral vein as a volume control prior to the bolus injection of the nanoparticles. Time was noted once the nanoparticles were flushed into the circulation with saline. Five millilitre venous blood samples were taken every 5 min post injection for the first hour and then every 20 min thereafter for 5 h. Blood was collected into heparinized tubes and stored at \(4^\circ\text{C}\) for later analysis. Following experimental procedures, animals were killed with a lethal intravenous injection of pentobarbitone and \(\text{KCL}\). Organs were harvested \((\text{spleen, pancreas, liver, heart, lungs, kidneys and brain})\) and stored in 10% buffered formalin for MRI, pEPR and histological biodistribution studies.

**The particle electron paramagnetic resonance technique**

Three biopsies were taken from each of the harvested lungs. Small PCR microtubes \((\text{Axygen, Fisher Scientific, Dublin, Ireland})\) were pre-weighed before and after addition of tissue to determine lung sample weight. Nanoparticle content in the lungs was measured using the pEPR technique \((\text{Pepric Particle Spectrometer (PPS3), PEPRIC nv, Belgium})\); values were expressed as mg/kg and compared across doses for each particle, MF66 and OD15. Nanoparticle content in blood samples \((150 \mu\text{L})\) at 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 180, and 200 min post particle injection were also determined using the pEPR technique. This technique is based on the application of EPR on the magnetic particle spin of the MNPs. The spin of the unpaired electrons within the iron oxide MNPs are put in resonance conditions, spins are aligned with a magnetic field of 10 mT and irradiated with an RF field of 300 MHz. The spin of unpaired electrons can be treated macroscopically as the overall spin of the MNP, with spin and magnetisation behaving as described by the Langevin function; adapted to describe the paramagnetic magnetization of the MNPs. This enables the excitation of the particle spin at low magnetic fields of about 10 mT. At these low fields, the resonant conditions only apply to the MNPs and not to the endogenous iron molecules present in tissue or blood, making pEPR a selective and direct detection method for MNPs. The detected electromagnetic RF signal induced by processing the tilted spins in excitation is proportional to the amount of particles, with a detection limit of 10 ng and a quantification limit of 35–70 ng, and therefore pEPR is a quantitative detection method for MNPs.\(^{10}\)
Magnetic resonance imaging

Scanning of ex vivo organs was carried out with a SIEMENS Avanto 1.5 T, 8 channel scanner (Mercy University Hospital, Cork, Ireland). Multiple organs (liver, spleen, heart, lungs, pancreas, kidney and brain) from each individual animal were scanned at the same time using a head coil. All image acquisition was acquired for qualitative inspection. Organs were wrapped in cling film and placed in the scanner, then the organs first underwent a 3-plane localizer sequence (TR/TE 20/15 ms, FA 20°, FOV 30 cm, 1 average, 15 slices, resolution: 192 x 256 μm/pixel, slice thickness = 5.0 mm) for visualisation. T2*-weighted gradient echo sequence (TR/TE 800/37 ms, FA 20°, FOV 250 cm, 4 averages, 20 slices, resolution: 256 x 256 μm/pixel, slice thickness = 3.0 mm) was inspected for nanoparticle distribution. Nanoparticle-treated organs were always referenced to control ‘blank’ (without nanoparticles) organs.

Histology

Five-micron thick sections were sectioned from multiple biopsies of each organ and mounted on polystyrene-coated glass slides (WVR International, Dublin, Ireland). Muscle sections were stained histochemically for qualitative iron oxide nanoparticle detection using Prussian blue stain. Sections were de-paraffinized in histoclear and hydrated with decreasing concentrations of ethanol to distilled water before being immersed in equal parts of HCL (Sigma Aldrich, Dublin, Ireland) and potassium ferrocyanide (Sigma Aldrich) for 20 min. Slides were washed in distilled water and counterstained with nuclear fast red (Sigma Aldrich) for 20 min. Slides were washed in distilled water before being dehydrated in a graded series of ethanol rinses. Slides were then cleared in histoclear and cover-slipped with DPX (Source Bioscience, Nottingham, UK) mounting medium.

Data analysis

To determine iron in histological sections, images (for a given study) were captured at low (×10) and high (×40) optical magnification on the same day as staining. All images were captured under the same pre-set lighting conditions using a BX51 Olympus microscope (Olympus Life Science Microscopes, Munich, Germany) and Olympus DP71 camera. Three to four sections were analysed from each animal. Data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using Graph Pad Prism software (GraphPad Software, La Jolla, CA, USA). All data was statistically analysed using Student’s unpaired t-tests or a one-way analysis of variance (ANOVA) with Newman–Keuls test where appropriate. In all tests, P < 0.05 was taken as significant.

ACKNOWLEDGEMENTS

This research was funded by the European Community’s Seventh Framework Program under grant agreement no. 262943; Multifunctional Nanoparticles for the Selective Detection and Treatment of Cancer (MULTIFUN). We thank our partners in CSIC and IMDEA for the preparation of the MNP’s, particularly Drs. Puerto Morales, Gorka Salas and Marzia Marciello, as well as Dr. Vijay Patel and Liquid Research Ltd. for the supply of MF66 MNPs. Surgivet Monitor was purchased with a grant from the Strategic Research Fund UCC 2012.

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