

Fe₃O₄/SALICYLIC ACID MAGNETIC NANOPARTICLES DELIVERY ROUTE. A SIMPLE METHOD FOR TISSUE TARGETING

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Abstract

Administration routes can greatly influence the pharmacokinetics, biodistribution and toxicity profile of the iron oxide nanoparticles. The purpose of this study was to investigate on a rat model the effect of three main administration routes of an aqueous dispersion of Fe₃O₄/salicylic acid magnetic nanoparticles (SaMNPs) on the pattern of their intravisceral storage. In order to identify the SaMNPs aggregates in the viscera we used Perl's Prussian blue stain. Specific tissue or cell targeting for SaMNPs deposits can be obtained by either of the three delivery systems employed in our study. The aerosol-mediated delivery is recommended for pulmonary and cardiac tissues targeting, the intravenous route is preferable for hepatic and renal tissues targeting and the intragastric administration is the recommended route for SaMNPs delivery to the hepatic, renal and cardiac tissues.

Rezumat

Calea de administrare poate influența semnificativ farmacocinetica, distribuția sau profilul toxicologic al nanoparticulelor cu oxid de fier. În acest studiu ne-am propus să analizăm, pe model murin, influența căii de administrare (inhalatorie, intravenoasă, intragastrică) asupra modului de acumulare intraviscerală a unei dispersii apoase de nanoparticule feromagnetice funcționalizate cu acid salicilic (SaMNPs). Pentru evidențierea depozitelor intraviscerale de SaMNPs am utilizat colorația cu albastru de Prusia. Acumularea țintită intratisulară și intracelulară a SaMNPs este specifică fiecărei căi de administrare utilizată în acest studiu. Administrarea inhalatorie se poate utiliza pentru acumularea țintită a nanoparticulelor în țesutul pulmonar și cardiac, calea intravenoasă poate fi folosită pentru țintirea parenchimului hepatic sau renal, iar administrarea intragastrică este utilă dacă se dorește țintirea ficatului, rinichiului și cordului.

Keywords: iron oxide nanoparticles, aerosol delivery, intragastric route, intravenous route, Prussian blue stain

Introduction

Lately, there has been a surge in the ferromagnetic nano-particle studies from the medical field, due to the fact that it was proven to be an attractive platform for bioapplications such as magnetic resonance imaging (MRI) contrast agents [24, 25, 27], hyperthermia [29, 45], vascular nanoblockage [33], tissue targeting [26] and targeted drug delivery systems [46]. In general, in order to accomplish a specific function, ferromagnetic nanoparticles must be engineered through a process called functionalization. This manufacturing technique consists of adding an organic or inorganic shell based on the specific function for which the nanoparticles are designed [49].

The biological behaviour of functionalized ferromagnetic nanoparticles is mainly influenced by the size of the nanoparticles [18] and the physico-

chemical properties of the shell [30]. Intravascular persistence of the nanoparticles is directly dependent on the diameter which is the main feature that causes the nanoparticles retention by the mononuclear phagocytic system (MPS) and their selective accumulation in different structures through endothelial fenestrations of the capillaries [5]. Additionally, the shell influences nanoparticles biodistribution by establishing their surface polarity and interaction with different structures of the biological system [19]. We have choose the salicylic acid coating based on recent studies that highlighted the anti-tumour effects of salicylic acid in breast, prostate, skin, lung, colorectal, oesophageal and liver cancers [1]. Salicylic acid acts through multiple mechanisms: inhibition of cyclooxygenase enzymes, cell apoptosis or antiangiogenic effect [7]. Iron oxide nanoparticles administration routes play a key role in bioavailability,

pharmacokinetics, biodistribution and toxicity [3]. Different routes for iron oxide nanoparticles administration have been used: topical [40], intraarterial [14, 12], intravenous [15], intrapulmonary [2], intra-tumour [17], *per os* [36], intraperitoneal [48], intra-vitreous [39], intramuscular and subcutaneous [38, 20]. In this paper we aimed to investigate the effect of the three main administration routes on the pattern of the SaMNPs intravisceral storage: aerosol-mediated delivery, intravenous and intragastric administration.

Materials and Methods

Ethical considerations

The current study was performed in conformity with the Guidelines for animal protection approved by the Ethics and Scientific Deontology Committee of the University of Medicine and Pharmacy of Craiova, Romania and according to international guidelines [11, 16].

Animals

A total of 9 female Wistar rats belonging to the Laboratory animal facility - Centre for Experimental Medicine of the University of Medicine and Pharmacy Craiova, Romania, with a body mass between 406 g and 435 g were used. The animals were held individually in polyethylene cages under controlled conditions of temperature (22 - 23°C), humidity (50 - 60%) and ventilation with a 12 hour light/dark cycle; they were allowed free access to a standard diet of pellet chow and water *ad libitum*. All rats were fasted overnight before the experiments. After assessing and confirming normal health status, the rats were randomly allocated to 3 groups of 3 rats in accordance with the study design.

Functionalized nanoparticles dispersion

The SaMNPs aqueous dispersion used in this work was synthesized by modified Massart co-precipitation as previously reported in our studies, and had a hydrodynamic diameter of 51.63 nm, a Zeta potential of +39.3 mV and an iron concentration of 0.356 mg/mL [32, 33].

Study design

Aerosol-mediated delivery: three rats were introduced into a custom-made nebulization system comprising an airtight chamber with a volume of 17.6 L connected by a plastic tube to a Chicco Ultrasonic Silent Aerosol Nebulizer that produces a dispersion of particles with a diameter between 3 and 5 µm. The rats were placed in the airtight chamber for 15 minutes and subjected to a continuous exposure to the aerosol medium by nebulizing a quantity of 25 mL SaMNPs suspension. At the end of the experimental time period, they were returned to their individual cages. Based on the quantity of nanoparticles suspension *per* given chamber volume and the allometric equations for the empirical relationship between minute volume and body weight [6], we

estimated an approximate intake of inhaled solution of 1.5 mL/rat.

Intravenous administration: three rats were anesthetized with Sevoflurane[®] and each received a single dose of 1 mL SaMNPs aqueous dispersion injected in the tail vein.

Intragastric administration: three rats were anesthetized with Xylazine[®] (9 mg/kg i.m.) and Ketamine[®] (80 mg/kg i.m.) and placed in a supine position. The hair was removed from the surgical site and the skin was disinfected with Betadine[®] solution followed by Lidocaine[®] 1% s.c. injection for local analgesia. The entry into the peritoneal cavity was obtained by a 1 cm left parasagittal incision of the abdominal wall. We identified the greater curvature of the stomach and a dose of 1 mL SaMNPs aqueous dispersion was given by intragastric injection close to the pyloric sphincter. The muscle layer of the abdominal wall was close to the continuous sutures (PDS 4-0 Ethicon) and interrupted sutures (Vicryl 4-0 Ethicon) were applied to the skin.

All 9 rats were euthanized after 24 hours; organs (lungs, liver, kidneys and hearts) were harvested and fixed in 10% neutrally buffered formalin for 72 hours. From our experience, 1 mL of aqueous nanoparticles dispersion dose used in the study for each rat is optimal because it does not induce hemodynamic changes after intravenous administration or gastrointestinal reflux upon intragastric administration. On the other hand, this dose contains 0.356 mg of iron, less than the daily intake required for a rat [31], which excludes an iron-induced toxic effect [47] in the case of the total loading of the SaMNPs suspension administered.

Tissue processing

The formalin-fixed tissue was embedded into paraffin wax, then sectioned into 5 µm thick slices on a HM350 rotary microtome coupled with a waterfall-based section transfer system (STS) (Thermo Fisher Sci., Walldorf, Germany), that allowed continuous cutting without losing any intermediate sections and mounted on glass microscope slides using the standard histological techniques. The slides were stained with routine haematoxylin-eosin (for tissue morphology study) and Perl's Prussian blue (for iron uptake evaluation). Perl's Prussian blue stain was used to identify the nanoparticle aggregates contained in the tissue samples. Perl's Prussian blue stain detects only the ferric iron (Fe³⁺) of the Sa-MNPs core.

Image analysis

All slides were analysed on a Nikon 55i microscope (Nikon GmbH, Wien, Austria) equipped with a 5 Mp cooled colour CCD camera and the Image ProPlus AMS image analysis package (Media Cybernetics, Bethesda, MA, USA), under constant contrast and illumination conditions.

Results and Discussion

The animals were continuously monitored throughout the experimental procedures. We did not observe

any signs of stress or major behavioural changes in the study subjects, such as psychomotor agitation or clinical signs of cardiac or respiratory impairment.

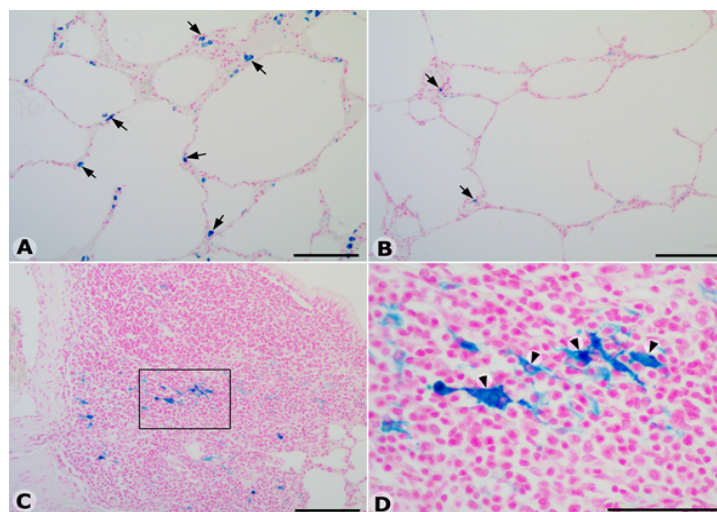


Figure 1.

Pulmonary tissue distribution of SaMNPs aggregates after aerosol mediated delivery (A, C, D) or intravenous administration (B). Histologic analysis highlighted intracytoplasmic deposits of SaMNPs in the alveolar (arrows A, B) and/or BALT macrophages (arrowheads C, D). Perl's Prussian blue staining. Bar = 100 µm (A, B, C); 50 µm (D)

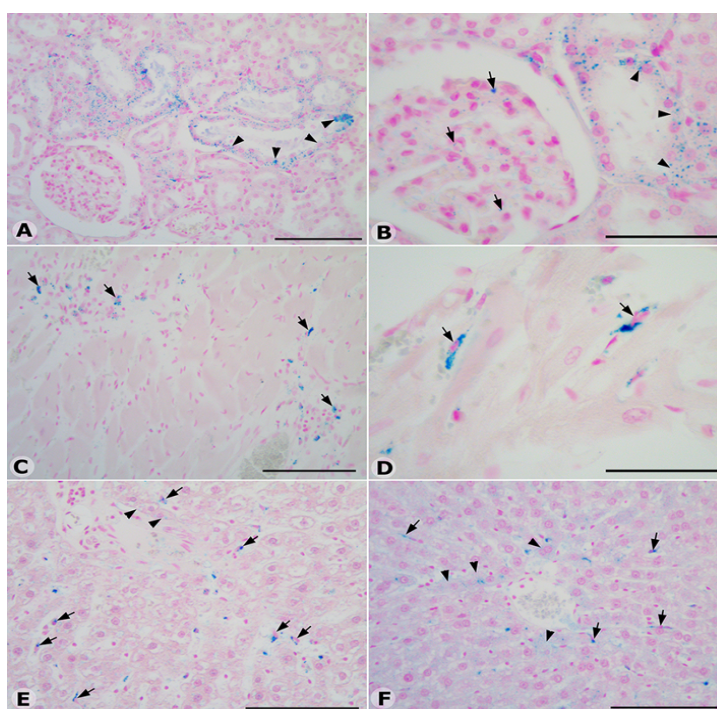


Figure 2.

Renal (A, B), cardiac (C, D) and hepatic (E, F) tissue distribution of SaMNPs aggregates after intravenous (A, B, C, D, E) and intragastric (F) administration. SaMNPs deposits in the glomerular (arrows) and proximal convoluted tubules (arrowheads) (A, B), cardiac macrophages (arrows) (C, D), Kupffer cells (arrows) (E) and hepatocytes (arrowheads) (F). Perl's Prussian blue staining. Bar = 100 µm (A, C, E, F); 50 µm (B, D)

The histological analysis of the tissue slides obtained from every studied viscera revealed a specific disposition of the functionalized SaMNPs aggregates highlighted with Perl's Prussian blue stain. In the pulmonary tissue, SaMNPs were identified as intra-

cytoplasmic deposits in the alveolar wall macrophages (Figure 1, A and B) and in the bronchial-associated lymphoid tissue (BALT) macrophages (Figure 1, C and D).

In the renal tissue, SaMNPs aggregates were identified in the cortical region with a particular distribution in the glomeruli, juxtaglomerular proximal convoluted tubules wall and in the interstitial tissue (Figure 2, A and B). We found no traces of SaMNPs deposits in the Malpighian pyramids or in the urinary tract which suggests that SaMNPs are completely reabsorbed at the proximal convoluted tubules level. In the heart tissue, we identified SaMNPs aggregates with a focal disposition in the interstitium and in

the macrophages surrounding the branches of the coronary arteries (Figure 2, C and D). In the hepatic tissue, SaMNPs in the form of intracytoplasmic deposits were identified in the hepatocytes and Kupffer cells (Figure 2, E and F).

Following the microscopic analysis of the studied viscera, we observed that the density of the SaMNPs, at the same dosage, is dependent upon the applied administration route (Table I).

Table I

Qualitative analysis of the SaMNPs deposits in relation to the administration route

Administration route	Dose (mL)	Lung		heart	kidney	liver
		BALT	alveolar macrophage			
Aerosol delivery	1.5	++++	++++	+++	++	+
Intravenous	1	++	+	+	+++	++++
Intragastric	1	N/A	N/A	++	+++	++++

N/A – not applicable

Aerosol-mediated delivery

Most of SaMNPs were observed in the pulmonary tissue. They had a uniform distribution in the alveolar septum macrophages of the pulmonary lobes in the lung periphery. In the central region of the pulmonary lobe the SaMNPs deposits are primarily present at the BALT level. Six lymphatic agglomerates of the ten studied contained SaMNPs deposits. The second most frequent viscera deposits were observed in the heart with foci of SaMNPs deposition in 6 perivascular areas out of 50 analysed. This preferential organ uptake can be explained by the fact that the heart is the first organ that receives the whole blood volume returning from the lungs where SaMNPs aggregates were absorbed. The third most common site for SaMNPs is the kidney, with fine deposits in 4 nephrons out of 50 analysed. The fewest SaMNPs deposits were observed at the hepatic parenchyma. Out of 10 hepatic lobules analysed, only 1 contained SaMNPs deposits in Kupffer cells.

Intravenous administration

Most SaMNPs deposits were identified in the liver with an intracytoplasmic distribution predominantly in Kupffer cells and scarcely in hepatocytes. All hepatic lobules studied contained SaMNPs deposits with the same pattern of distribution. The renal SaMNPs deposits showed a relative uniform distribution in the cortex. Out of the 50 nephrons analysed, 43 contained SaMNPs deposits in the regions near the glomeruli. In the heart we identified perivascular SaMNPs deposits foci with almost the same density found in the aerosol-mediated delivery. Out of 50 perivascular areas analysed, 8 presented foci of SaMNPs deposits. In the lungs we identified few macrophages with intracytoplasmic SaMNPs deposits with a uniform pattern of distribution in the central and peripheral pulmonary tissue. All 10 BALT analysed contained SaMNPs deposits.

Intragastric administration

Similar to the intravenous administration, most SaMNPs deposits were found in the liver predominantly in hepatocytes and scarcely in Kupffer cells of the centrilobular region. The kidney SaMNPs deposits showed a cluster-like distribution in the cortex. In some areas of the cortex, out of 50 nephrons analysed, 46 contained SaMNPs deposits in the regions near the glomeruli whereas in other areas only 4 of 50 nephrons analysed contained SaMNPs deposits in the regions near the glomeruli. In the heart we found the same distribution of SaMNPs deposits as described in the aerosol-mediated delivery. As we could not exclude gastro-oesophageal reflux and possible SaMNPs bronchial aspiration, the lung was not included in the study when the intragastrical route of administration was used.

The key factors in nanoparticle biodistribution are the size, shape and shell characteristics [10]. Regardless the administration route employed in our study, we observed the nanoparticles passage into the blood stream. Previous studies have shown that once the nanoparticles reach the blood stream they are primarily taken up by monocyte phagocyte system (MPS) [28]. Therefore nanoparticles smaller than 10 nm readily pass through the intercellular junctions and are quickly eliminated by glomerular filtration due to the 10 nm pore size of the glomerular basal membrane [22]. Nanoparticles with a diameter less than 50 nm have been observed in the lymphatic tissue and in the MPS macrophages [28], those with a diameter between 50-150 nm are retained by Kupffer cells and the ones with a diameter above 200 nm are retained in the spleen [5]. Schweitzer *et al.* [42] point out the fact that nanoparticles with a diameter between 20 and 100 nm tend to accumulate in the bone marrow, probably due to the high level of sinusoidal microcirculation permeability [23, 34].

One of the factors that can partially explain the nanoparticle transvascular passage is the different

types of tissue blood capillaries. For example, the glomerular membrane has non-sinusoidal fenestrated blood capillaries with open *fenestrae* with pores that have an upper limit of 6 - 12 nm and additional slit diaphragms that restricts the filtration of particles upward of 6 nm. The lung capillaries are non-sinusoidal non-fenestrated with loose junctions (*macula occludens* interendothelial junctions) that restrict the passage of nanoparticles larger than 5 nm, while the intestinal mucosa has non-sinusoidal diaphragmed *fenestrae* with a physiologic upper limit of pore size between 6 - 12 nm. On the other hand, the rat liver has sinusoidal blood capillaries with open *fenestrae* with a transvascular flow around 280 nm [41]. However, due to the fact that we used SaMNPs with a hydrodynamic diameter of 50 nm, the route of non-restricted transvascular passage through endothelial fenestrations is limited to the hepatic and bone marrow sinusoidal blood capillary. All the aforementioned data does not explain the presence of the SaMNPs deposits that we observed in the other studied viscera. Therefore there must be additional pathways involved in the nanoparticles absorption and biodistribution.

The interaction between the SaMNPs and the MPS in organs with a sinusoid-type circulation such as the liver, spleen and bone-marrow can be considered responsible for the visceral pattern of nanoparticles distribution. Non-resident SaMNPs loaded macrophages from these tissues can re-enter the blood stream and travel freely in the body to reach other viscera. In addition, the significant extravasation (55%) of monocyte in the lymphoid tissues [4, 13] and continuous renewal of the cardiac macrophage pool with circulating monocytes [43], can provide an explanation for SaMNPs loaded monocyte-derived macrophage highlighted in the BALT or cardiac tissues.

The biological properties of the salicylic acid coating contribute to the tissues-distribution pattern of the SaMNPs. It has been shown that salicylic acid shell provides a negative charge [32] that increases the SaMNPs cell uptake [19]. Furthermore, it promotes the cellular absorption by increasing the plasma membrane permeability [21].

When the aerosol-mediated delivery is used the lung is the first organ exposed to the entire quantity of the inhaled nanoparticles, and thus the SaMNPs uptake is primarily at the alveolar level, in the form of intracellular deposits in the resident macrophages. A possible uptake by non-resident macrophages that can transport the nanoparticles at the BALT can explain the SaMNPs deposits revealed at this site. Through the lymphatic route, the nanoparticle loaded macrophages re-enter the lungs and then reach the heart through the coronary arteries where they are partially retained. The remainder of the circulating

nanoparticle loaded macrophages can further reach the kidneys and the liver.

In the case of intragastrical delivery, SaMNPs pass the intestinal epithelium by passive transport facilitated by the salicylic acid shell; enter the portal vein to reach the hepatic parenchyma. Thus, the liver is the first organ to receive the entire amount of absorbed nanoparticles. As revealed by some of us (data in press), the SaMNPs pass through the Disse space and are taken up primarily by the hepatocytes. Given that the liver produces large quantities of lymphatic fluid and that lymphocytes can translocate from the sinusoid capillary blood to the hepatic lymph [35], SaMNPs can also employ this route of circulation. It is interesting to point out that in this case the resident Kupffer cells showed a modest uptake of nanoparticles.

The results revealed that the intravenous administration route of the SaMNPs is more complex. Thus, the SaMNPs administered in the tail vein travelled freely in the blood stream and reached the viscera *via* arterial supply by passing the hepatic filter. Given that the amount of nanoparticles retained in the heart is approximately the same as in the other routes of administration, we can assume the fact that the SaMNPs are not retained directly in the heart cells by this first organ passage. This route of administration possibly allows to a greater quantity of nanoparticles to reach the bone marrow. At this level they can be scavenged by the cells of the MPS and released in the blood stream. The MPS cells present in the liver, lymph nodes, lung and bone marrow [44] play an important role in this route of nanoparticles administration [3]. Others have demonstrated that iron oxide nanoparticles with a diameter between 10 nm and 100 nm have an extended half-life that maximizes their potential to reach organs such as lymph nodes [9, 37] and arterial wall [8].

The SaMNPs deposits around the renal glomeruli and their absence in the distal convolute and collector tubes can be considered a proof of the beneficial and synergic effect of the salicylic acid shell. This shell provides a negative charge which can lower blood clearance by the macrophages [3] and promotes the renal reabsorption.

Conclusions

To our knowledge, this is the first study which tested three different administration routes for the same nanoparticle suspension. Our data showed that regardless the administration route, SaMNPs reached the blood stream. A correlation between the delivery route and pattern of the SaMNPs deposits on the studied viscera has been observed. Thus, the aerosol-mediated delivery is recommended for pulmonary (BALT and alveolar macrophages) and cardiac tissue targeting, with minimal hepatic accumulation. The

intravenous route is preferable for hepatic (Kupffer cell) and renal (uniform distribution) tissues targeting. The intragastric administration is the recommended route for SaMNP's delivery to the hepatic (hepatocytes), renal (cluster-like distribution) and the cardiac tissues. Therefore, specific tissue or cell targeting for SaMNP's deposits can be obtained by using one of the three delivery systems employed in our study.

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