

UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA  
DOCTORAL SCHOOL

**PhD THESIS SUMMARY**

**IRON OXIDE NANOPARTICLES WITH  
CITRIC ACID COATING: PROPERTIES AND  
STUDIES ON EXPERIMENTAL MODELS**

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## INTRODUCTION

One may say that magnetic nanotechnology represents the future of science. Others have defined nanotechnology as “the understanding and control of matter at dimensions of roughly 1-100 nanometers ( $10^{-9}$  to  $10^{-7}$  m), where unique phenomena enable novel applications” according to the National Nanotechnology Initiative (NNI) of United States [1].

In the past decade, magnetic iron oxide nanoparticles (MNP) were the only magnetic nanomaterials approved for clinical use by the United States FDA (Food and Drug Administration) [2,3].

MNPs have many important biomedical applications such as drug carriers, contrast agents, cancer treatment, gene therapy, hyperthermia, biosensors and most recently, they have demonstrated a new role in cognitive function of the human brain [4,5].

While naked magnetic nanoparticles have a hydrophobic nature, they are not stable in normal physiological conditions and tend to form aggregates. In water based magnetic fluids, the magnetic phase (mainly iron oxides) requires different coating materials that give them stability and compatibility with biological fluids [6,7].

Coating materials used to sterically stabilize the iron oxide nanoparticles often contain carboxyl functional groups. These functional groups are popular because they form stable covalent linkage between coating agents and iron oxide surface.

## BACKGROUND

This part consists of the following six chapters in which we presented data from the literature (articles, manuscripts, monographs).

In the first chapter we presented general characteristics of different types of magnetic nanoparticles, in particular highlighting iron oxide nanoparticles.

In the second chapter we discussed about nanoparticles magnetism. We presented different types of magnetism that nanoparticles can manifest depending on their structure, size and the coating material, namely: ferromagnetism, superparamagnetism and paramagnetism.

The third chapter was a review of nanoparticles synthesis. We detailed the most important methods: lithography, co-precipitation, microemulsion, hydrothermal synthesis, thermal decomposition, pyrolysis and plasma synthesis, highlighting the advantages and disadvantages of each.

In the fourth chapter we detailed the most important techniques for the detection, measurement and characterization of nanoparticles.

In the fifth chapter we presented the most important and successful applications of nanoparticles in medicine. Recent research shows the use of magnetic nanoparticles in imaging, hyperthermia, drug delivery, biomedical treatments, etc.

In the sixth chapter we described the peculiarities of hemostasis. Medical nanomaterials can act either as procoagulant agents or as hypocoagulant agents, depending on the size, coating and chemical composition.

## PERSONAL CONTRIBUTIONS

### CHAPTER VIII. Synthesis and characterization of magnetite nanoparticles coated with citric acid (MA-COOH)

The synthesis of Fe<sub>3</sub>O<sub>4</sub> (magnetite) nanoparticles coated in citric acid was made by co-precipitation reaction as follows: 2.7 g FeCl<sub>3</sub>·6H<sub>2</sub>O and 4.0 g FeCl<sub>2</sub>·4H<sub>2</sub>O were dissolved in 200 ml of ultrapure water. Then the mixture was stirred vigorously until iron salts were completely dissolved. Subsequently, to the mixture was added rapidly 10 ml of ammonium hydroxide at room temperature. Vigorous stirring was continued for 15 min at 75 °C. After the reaction was completed, the resulting black product was collected on a permanent magnet and washed three times with ultrapure water. To avoid the agglomeration of magnetite nanoparticles, it was added 150 ml of 10 wt% citric acid aqueous solution and stirred for 15 minutes at 60 °C.

Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles modified with citric acid (MA-COOH) were magnetically separated and washed three times with ultrapure water to remove impurities

MA-COOH aqueous suspension was analyzed in terms of dimensional aspect using Dynamic Light Scattering technique and in terms of colloidal stability using zeta potential technique with a Brookhaven 90 Plus device. The results indicate a uniform distribution, being present only a single particle size range and most nanoparticles are of 9.5 nm size, and zeta potential has a value determined in the range 27-29 mV, which means that the suspension has moderate stability [8,9].

The dried nanoparticle sample was chemically analyzed by X-ray photoelectron spectroscopy (XPS) and magnetic analyzed by Magneto-optic Kerr effect magnetometry (MOKE) in collaboration with The National Institute of Materials Physics (NIMP-Bucharest).

Chemical analysis of MA-COOH by XPS was performed in an analysis enclosure of a cluster of surface and interface science score Specs GmbH under vacuum UHF, with pressure values basic order of 1-2×10<sup>-9</sup> mbar and values during the measurement of 3-5×10<sup>-8</sup> mbar. It is distinguished during the analysis from the spectra and the energy values related to the line photoemission that the chemical formula is respected, Fe appears as oxidized in the specific position of Fe from magnetite and the other chemical elements appear to values which are consistent with international data bases values for compounds such as citric acid.

For MOKE analysis it was used an AMACC Anderberg and Modéer Accelerator AB system, permitting exposing the sample to a magnetic field of up to 0.55 T (437.68 kA/m). The analysis showed that the nanoparticles present magnetic properties.

## **CHAPTER IX. The study of in vivo biological properties of MA-COOH on experimental models**

Due to the fact that a suspension of magnetic nanoparticles in an artificial physiological environment, could not fully simulate different cells of the biological system, we focused on evaluating the pharmacokinetics, biodistribution and the influence on hemostasis of nanoparticles in a in vivo situation, using a murine model. The murine model remains one of the best models for biomedical study, thanks to several features and benefits as follows: small dimensions, similarities to humans and the whole genome sorted by similarities in humans [10].

### **The experimental model used to study the kinetics of elimination of MA-COOH in blood**

There were used 10 male rats Sprague Dawley (two for control purpose) with an average weight of 400 g and age of ninety days. The animals were weighed then anesthetized with a mixture of Xylazine 10mg/kg and Ketamine 100 mg/kg intraperitoneally administrated. Then with the help of a syringe connected to a catheter, it was injected in the jugular vein, a volume V ( $\mu$ l) equivalent to the concentration of 15  $\mu$ mol Fe/kg biocompatible ferrofluid, consisting of magnetic nanoparticles of Fe<sub>3</sub>O<sub>4</sub> coated with citric acid, diluted in an isotonic glycosylated solution, to a final volume of 0.5 ml. After time periods of 0, 30, 90, 150, 240 minutes and a day, blood samples were taken from the tail vein. The ambient temperature was maintained in the interval of 24-27 °C. The blood was collected using a syringe with a 23G needle inserted directly in the blood vessel and was stored in cap Eppendorf tubes at the temperature of -80 °C. After completing the blood sampling, the bleeding was stopped with a mixture of silver nitrate and applying pressure [11].

After injection, the rats were recovered in lateral decubitus in a cage with shavings. They were observed at least 4 hours until no signs of pain appeared and then once per day.

The kinetic of elimination of MA-COOH from the bloodstream was determined by using EPR spectrometer electron paramagnetic resonance X-band Bruker ELEXSYS E580 model equipped with a resonant cavity type Super High QE ( SHQE ) 4123SHQE ER model (NIMP-Bucharest).

### **Experimental model used to determine MA-COOH biodistribution in organs**

There were used a group of 10 Sprague Dawley rats. At 24 hours after intrajugular administration of MA-COOH, similar to the above described procedure, the animals were euthanized with 120 mg/kg of sodium pentobarbital intraperitoneally administered. The rats were euthanized and then perfused transcardially with saline solution by introducing a 23G needle, connected to a saline container located at 50 cm height from the dissecting table, in the left ventricle. Samples of tissue from the liver, pancreas, brain, muscle, kidneys were collected and frozen at -80 °C. Then the samples were homogenized and lyophilized to determine the concentration in organs by electron

paramagnetic resonance. Control samples were collected from two rats which were not injected with the nanoparticles suspension.

Histopathological analysis was performed using Prussian blue staining, which contains a mixture of 20% hydrochloric acid and 10% potassium ferrocyanide solution in the volume ratio of 1:1 (Corp. Merck KGaA, Darmstadt, Germany) to observe the tissue distribution of MA-COOH. Hydrochloric acid degrades MA-COOH in ferric ion, which will react with potassium ferrocyanide, forming  $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$  (ferric ferrocyanide), a blue precipitate, insoluble in organic solvents. Various organs were collected (liver, spleen, pancreas, kidney, brain and muscles) by surgical methods presented in the previous chapter. Samples of organs were transferred to labeled containers containing an appropriate amount of 10% neutral buffered formalin (NBF).

Paraffin embedded tissues, of the rats which were administered MA-COOH, were stained with a solution of Prussian blue and then washed with PBS (Phosphate-buffered saline).

### **Experimental model used to determine MA-COOH influence on hemostasis**

Knowing that nanoparticles used in our study are coated with molecules of citric acid and due to the fact that citric acid is a chelator agent for calcium ions, we tried to quantify potential changes in the blood coagulation cascade.

In this regard, we decided to evaluate these alterations in hemostasis by means of simple laboratory tests (clotting time and bleeding time), but also using a new imaging method, experimental, optical coherence tomography, recently used by our research group to evaluate whole blood coagulation.

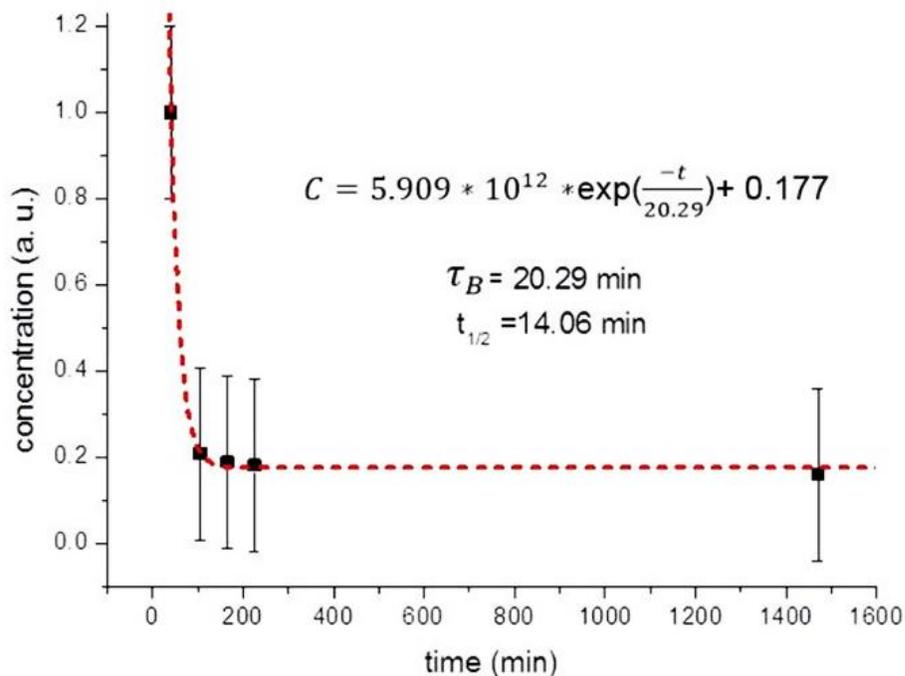
## **CHAPTER X. RESULTS**

### **Electronic paramagnetic resonance analysis results**

The quantitative EPR method determined the absolute number of electron spins, which contributes to the EPR signal. In this case, this number is equivalent to the number of  $\text{Fe}^{3+}$  ions in the lyophilized blood amount, introduced into the EPR tube.

The EPR line intensity at  $g=2.1$  was found to be proportional to the concentration of nanoparticles and optimum temperature for spectra acquisition is the room temperature ( $T=298\text{ K}$ ) [12].

The half-life associated with the nanoparticles elimination process in the blood circulation is given by  $t_{1/2}=14,06\text{ min}$ .



*Blood concentration– time curve following intravenous administration of MA-COOH.*

Magnetic nanoparticles are not delivered only to the liver, but they are also transferred to other organs of the rat [13].

For the quantitative determination of MA-COOH concentrations in organs it was used the routine for calculating the absolute amount of spins, included in the Bruker XEPR program, based on the double integration of EPR spectra.

Considering the concentration of nanoparticles in the sample at 30 minute after MA-COOH administration, as the reference concentration,  $C_{REF}$ , the magnetic nanoparticles had the following organ concentrations, measured with EPR:

Liver:  $(120 \pm 24) C_{REF}$

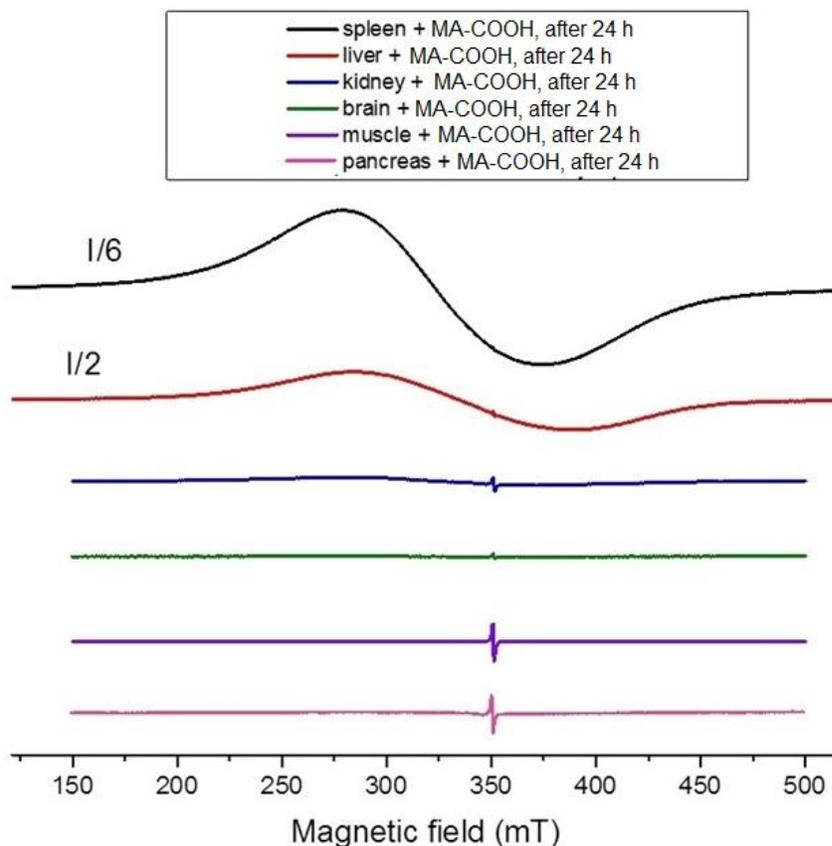
Spleen:  $(706 \pm 141) C_{REF}$

Kidney:  $(3.3 \pm 1.5) C_{REF}$

Pancreas:  $(0.7 \pm 1.5) C_{REF}$

In brain and muscle the MA-COOH concentrations were approximately zero.

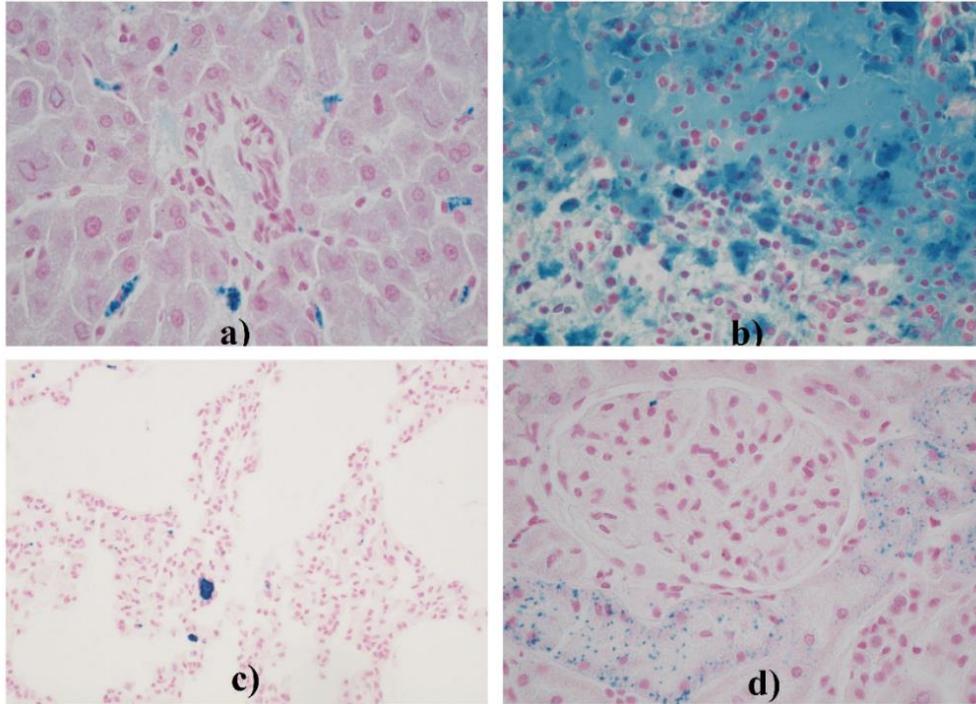
Due to the much smaller mass of the spleen, the absolute concentration of nanoparticles is higher in spleen than in liver.



*EPR spectra at room temperature of different lyophilized rat organs at 24 h after MA-COOH administration*

### **Histopathological analysis results**

Histopathological assessments confirmed all the deposits, using Prussian Blue staining. As expected, in spleen were found nanoparticle deposits in and out of the macrophages, especially in the red pulp. In liver, some deposits were noted in the periphery of the capillary sinusoids and also in Kupffer cells and Ito cells. Smaller deposits were found in the portal and centrilobular spaces. As for the kidneys, MA-COOH were primarily located within the proximal convoluted tubules and to a lesser extent in the distal convoluted tubules, more precisely in the epithelial cells which cover the collector tubes.



*Deposits of MA-COOH in a) liver, b) spleen, c) lungs, d) kidney of rat sacrificed 24 hours after intravenous administration of MA-COOH. Prussian blue staining 40x*

### **Results of MA-COOH influence on hemostasis**

After analyzing coagulation times we noticed a minimum of 180 seconds and a maximum of 240 seconds for the test group with a mean value of  $211,5 \pm 4,731$  (SD) seconds. There was a slight increase in clotting time compared to the control group ( $205,5 \pm 4,229$ ), but statistically insignificant ( $P= 0,3503 > 0,05$ )

After analyzing bleeding times we observed a minimum of 285 seconds and a maximum of 360 seconds for the test group with a mean value of  $324,8 \pm 5,888$  (SD) seconds. There was a slight increase in bleeding time compared to the control group ( $315,8 \pm 5,807$ ), but statistically insignificant ( $P= 0,2833 > 0,05$ ).

In addition, our results demonstrate that the blood OCT tomography images of the two groups (test and control) are similar, which means that the MA-COOH does not cause significant abnormalities in the coagulation process.

## **DISCUSSIONS**

Citric acid molecules on the surface of the nanoparticles may be used in surface functionalization by conjugation with biomolecules in a number of biomedical applications, such as drug transport systems, bioseparation, contrast enhancement for magnetic resonance imaging etc.

Previous studies have shown that most of nanoparticles introduced into the bloodstream are taken up by the organs of the mononuclear phagocyte system (MPS) such as liver and spleen.

It is known that iron oxide nanoparticles are taken up by endocytosis in MPS by Kupffer cells in the hepatic sinusoids and by macrophages in the red pulp of the spleen, and are then degraded in the lysosomes of these cells.

Degraded iron is eventually removed or reused inside the body through metabolic pathways of iron, products of degradation of Fe (II) and Fe (III) then becoming part of hemoglobin and of various proteins and binding chelators of iron (ferritin and transferrin) [14,15].

The results obtained from pharmacokinetic analysis and tissue distribution were consistent with the study of Gamarra et al. [12] in which the half-life of dextran coated magnetite nanoparticles with diameter of 80-150 nm was about 11-12 minutes and their accumulation in liver was observed after EPR analysis.

Also, Gu et al. [16] evaluated the biodistribution and degradation of iron oxide nanocrystals coated with polyethyleneglycol in mice. The iron accumulated in organs at 24 h after intravenous injection (5 mg Fe/kg) of Feridex and nanocrystals (5-30 nm) confirms the results obtained in this study.

The reason for which we have studied the influence of MA-COOH on hemostasis is that citric acid is a calcium chelator which, in ionic form, is a cofactor for a number of coagulation factors. Data about the effect of nanoparticles in the coagulation process are limited, but some studies have showed that they have an anticoagulant effect [17,18]. However, such experiments cannot be compared to ours, as the nanoparticles were either uncoated or they had different structure. In our case, the dose of MA-COOH injected in mice was low, therefore the anticoagulant effect was statistically irrelevant.

## CONCLUSIONS

1. After using co-precipitation method, there were synthesized iron oxide nanoparticles coated with citric acid, whose structure was confirmed by specific methods: XPS spectrometry, optical magnetometry MOKE, Dynamic Light Scattering, zeta potential.

2. The synthesized nanoparticles and tested in further studies had an average diameter of 9.5 nm and a zeta potential of 29 mV, characteristics which give them a good stability of the aqueous dispersion.

3. Following intravenous administration of nanoparticles in murine model, it was observed a rapid capacity of elimination from the bloodstream, the nanoparticles half-life being 14.06 minutes.

4. Concerning the intravascular behavior (intravenous jugular), nanoparticles have shown a significant accumulation especially in liver and spleen, both organs having a special capillary flow (sinusoidal) and a rich population of capable phagocytic cells.

5. Nanoparticles influence on hemostasis was one of slight increase in clotting and bleeding times, but not statistically significant .

6. In conclusion, nanoparticles used in our study, with a trophism for organs like spleen and liver, can be easily functionalized (having coating rich in carboxyl groups) and may be used in future tests that can target pathologies of these two organs.

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