UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

PhD THESIS SUMMARY

IRON OXIDE NANOPARTICLES WITH POLAR SHELL: PROPERTIES AND BIOLOGICAL PERSPECTIVES

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CONTENTS

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Background

Nanotechnology is the science that studies the characteristics and applications of nanosized materials. Nanoparticles are amorphous or crystalline particles with at least one dimension smaller than one micron. They have a surface that can act as a carrier for the droplets of liquids or gases (1). Some applications of nanoparticles in the biomedical field are: release of medical substances and genes, detection of proteins and pathogens, the research of the structure of DNA, fluorescent biological markers, separation and purification of cells and biomolecules, phagokinetic studies, destruction of tumors using heat (hyperthermia) (2).

Magnetite nanoparticles are the most studied nanobiomaterials. There are various methods to synthetize nanoparticles with diameters between 2-50 nm with specific magnetic properties and low toxicity on the human body shown through numerous studies *in vitro* and *in vivo* (3). Magnetic nanoparticles with diameters between 50-100 nm cannot cross the vascular wall and are metabolized in the liver by Kupffer cells, while those with diameters more than 200 nm are retained by the splenic capillaries (4). Due to their superparamagnetic properties, the ferrite nanoparticles with diameters around 50 nm size are the most studied (5). An appropriate design of nanoparticles and the unique properties of nanosystems may lead to their use as a theragnostic agent, combining two directions of applicability: therapeutic and diagnostic (6).

With the development of the pharmaceutical industry oriented towards new molecules for controlled release systems, the potential for using the chick chorioallantoic membrane has increased. These are referred to as CAM methods or *in vivo* CAM models (7). Chicken embryos are used for evaluating activity, cytotoxicity, pharmacokinetics, biodistribution and biocompatibility of drugs. According to United States Food and Drug Administration (FDA Guidance for Industry, June 2006), some replace the use of preclinical studies.

Many studies mentioned different applications of tissue engineering in the study of CAM model (8), biomaterials and implants (9), wound healing (10), angiogenesis and antiangiogenesis (11), in tumor cell invasion and metastasis (12), intestinal cancer (13), glioma (14), cancer of the prostate (15), leukemia (16), osteosarcoma (17), ovarian cancer (18).

The blood vessels are formed through two mechanisms (19):

• Vasculogenesis - the process of direct formation of blood vessels as a result of the in situ differentiation of precursor cells of angioblast cells;

• Angiogenesis - the formation process of new vessels from preexisting vessels, capillaries and postcapillary venules.

The chick CAM is used in the study of the macromolecules with angiogenic and antiangiogenic effects on tumor cells (20). At 72 - 96 hours after angiogenic stimulation, an increased vascular density with radial arrangement around the tumor implantation site is observed, and, after stimulation with antiangiogenic component, a decrease in vascular density or loss of vascularization is observed (152).

Personal Contributions

Chapter IV. Synthesis and characterization of Fe_3O_4/salicylic acid nanoparticles

The synthesis process of nanoparticles of Fe_3O_4/salicylic acid has the following steps (21):

1. Preparation of Solution 1: 10 g KOH, 0.5 g of salicylic acid and 250 mL of ultrapure water;
2. Preparation of Solution 2: 2 g $FeCl_3$, 1.25 g $FeSO_4$ and 250 mL of ultrapure water.
3. Solution 2 was slowly added to solution 1 and the resulting mixture was allowed to stand for 30 minutes at 50^0C.
4. The mixture was then subjected to magnetic separation using a powerful NdFeB magnet, Q51-51-25-N, Moe 38-40;
5. Repeated washing with ultrapure water, followed by magnetic separation;
6. Stirring for 10 minutes in a medium of KOH 30% (ionization step);
7. Repeated washing with ultrapure water, with magnetic separation;
8. Fast dispersing in ultrapure water under the action of the magnetic field;
9. Filtration through a sieve of polytetrafluoroethylene with a diameter of 22 μm.

Concurrently, Fe_3O_4 nanoparticles were synthesized similarly, but without adding salicylic acid in the solution 1. Synthesis was completed with a final step of drying the obtained nanoparticles at 120^0C for 12 hours.

Sample analysis was performed by XRD using a Rigaku Ultima IV spectrophotometer with Bragg-Brentano geometry, angles in the range 10^0-100^0, with step set to 2s and $0,05^0$, with CuKα source. Comparative analysis of X-ray diffractograms of iron oxide nanoparticles found characteristic peaks of magnetite.

In the FT-IR spectra, the characteristic absorption band of magnetite was observed at 550 cm^{-1} and the specific absorption bands of nanoparticles with salicylic acid shell at: 1557, 1411, 1329, 1026 cm^{-1} (specific salicylate band). There was an absorption band characteristic of carboxyl group present at 1657 cm^{-1} in the spectrum of salicylic acid, at 1617 cm^{-1} in the iron (III) salicylate spectrum and at 1,557 cm^{-1} in the frequency spectrum of the iron oxide nanoparticles with salicylic acid shell.

TEM analysis was performed using an electron transmission microscope with high resolution Tecnai G2 F30 S-TWIN TM (FEI Company, Hillsboro, OR, USA). Microscope operating mode was set to 300 kV with point resolution of 2 Å and 1 Å line resolution. Analysis of the samples shows that after synthesis nanoparticles of Fe_3O_4/salicylic acid were obtained with an average diameter of 60 nm, dispersed and with spherical shape.

The particle size distribution and determination of the value of the Zeta potential were carried out by Dynamic Light Scattering method (DLS) using a Brookhaven 90 Plus device. The results indicate a homogeneous particle size distribution for each group studied. For batches with a nanoparticle size below 210 nm, the Zeta potential determined has a value of over 40 mV, which is proof of the long-term stability of the aqueous dispersion of Fe_3O_4/salicylic acid.

Chapter V. Study models for the investigation of the biological properties of Fe_3O_4/salicylic acid nanoparticles

The murine model is one of the most study models for cancer development and treatment. This model has several features and benefits as follows; small size, resemblance to human models due to similarly ordered genome (22), easy testing of new drugs, easy implantation of tumor grafts and rapid development of tumors, relatively low cost, etc.

In recent years, medical research has been oriented also on using the chick CAM as an *in vivo* model, especially for studying different mechanisms involved in tumorigenesis. The characteristics of this model allow describing both the microenvironment of the tumor, the interaction between tumor cells and stromal tissue, as well as the stages of dissemination of tumor cells of different types: glioma, colorectal carcinoma, fibrosarcoma, etc. (23). These arguments grounded the choice for the chick CAM as one of the *in vivo* models of the compatibility and dispersion action of iron oxide nanoparticles with salicylic acid shell.

Implantation of the murine melanoma tumor xenografts

In 8-days-old chicken embryos, CAM is already formed and its vessels are in a stage of angiogenesis by intussusception. During this stage, tumors implanted into the surface of the membrane undergo rapid vascularization.

For implantation of tumor xenografts, the eggs are removed from the incubator and are placed on an insulated support (polystyrene). After removing the adhesive tape from the window the chicken embryo viability and the development stage of the chick CAM were examined using a Zeiss stereomicroscope with side illumination.

If the chick CAM was not sufficiently developed or abnormalities in the development of the embryo were observed, the egg was removed from the experiment. If the chick CAM was normally developed, its surface was inoculated under sterile conditions using a syringe needle with a 0.1 mL of tumor tissue, aspirated from the melanoma developed in the murine model.

After completion of the inoculation with the tumoral tissue, the eggs were returned to the incubator and monitored daily using a stereo microscope until day 16 of development, when all tumor xenografts and surrounding chick CAM were harvested and fixed in 10% formalin neutral solution for subsequent histological processing.

Determining the bioavailability of the iron oxide nanoparticles with salicylic acid shell

Determining the systemic circulation time of the aqueous dispersion of ferromagnetic nanoparticles with salicylic acid shell for the chicken embryo is an essential step for assessing the

bioavailability of the nanoparticles. Circulation time is required for the design of an *in vivo* experiment, to present the use of these nanoparticles. To carry out this experiment, three batches of chicken embryos at day 12 of development were used. At day 12, the chorioallantoic membrane vessels are well developed and the intravenous injection is possible under optimal conditions.

From the analysis of data on the circulation time of ferromagnetic nanoparticles with salicylic acid shell in the vascular system of the chicken embryo, the following observations can be made:

1. for MNP injected doses, less than 0.1 mL, there is a sharp downward trend in the amount of circulating nanoparticles; images acquired after 5 minutes exposure of the static magnetic field action and at 90 minutes before injection do not emphasize the ferromagnetic nanoparticles with salicylic acid shell accumulated on the inner wall of the arteriole;
2. for injected doses of ferromagnetic nanoparticles with salicylic acid shell above 0.1 mL, there is a tendency for sudden decrease in the amount of circulating nanoparticles in the first 120 minutes, until achieving a constant circulating concentration of nanoparticles, which then modifies very little over long periods of time (over 15 hours).

Biocompatibility study of Fe_3O_4/salicylic acid nanoparticle dispersion

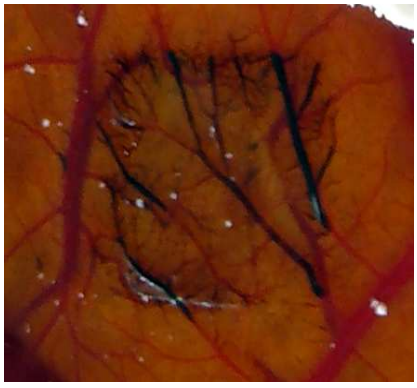
Determination of the biocompatibility dispersion of ferromagnetic nanoparticles with salicylic acid shell was performed *in vivo* chick CAM model by studying its effect on the normal development of the embryo. In this study were used three groups of 5 chicken embryos at day 11 of development, of which one was the control group. A number of 5 embryos (group I) were injected with a single dose of 0.2 mL of the dispersion solution of ferromagnetic nanoparticles with salicylic acid shell, and the other five embryos (group II) with two consecutive doses at an interval of 24 hours. After injection, all the eggs were returned to the incubator and examined daily using a stereomicroscope equipped with a DCM 510 camera, until normal hatching. All injected embryos developed normally and survived, without any abnormalities, hatching as expected and growing normally until four months.

***In vivo* study of ferromagnetic nanoparticles intravascular behavior on the CAM model**

For this experiment, we used a group of 15 chicken embryos at day 11 of development. Ten chick embryos were injected with 0.2 mL of an aqueous solution of ferromagnetic nanoparticles with salicylic acid shell at a concentration of 0.19%. Five other embryos were used as control group.

After injection, on the chick CAM of all embryos, injected or uninjected, a NdFeB magnet with the strength of 0,18T was applied for 30 minutes. The embryos were then returned into the incubator. After 7 days from the injection, five embryos were sacrificed and fixed in 4% neutral formalin solution, after which the chick CAM were collected for histological processing using standard methods. The accumulation of ferromagnetic nanoparticles aggregate in the area were the static magnetic field was applied is observed at the level of arterioles and venules of the chick CAM of the injected embryos.

Nanoparticles aggregates were accumulated only in the vessels that came in contact with the magnet. Profound vessels of the vitelline circulation were not blocked by accumulating nanoparticles, at this level the magnetic field being unable to block nanoparticles aggregates and therefore rapidly removing them from the bloodstream. These findings indicate the potential of nanoparticles dispersions to block only vessels in a particular area of interest, by adjusting the magnetic field intensity at the volume of the vessels and local hemodynamic conditions.



Intravascular accumulation of ferromagnetic nanoparticles under the action of a static magnetic field of 0,18T applied for 30 minutes

The lack of nanoparticles aggregates in vessels outside the magnet's action shows that the aggregates are dispersed quickly and do not block downstream capillaries. This is proof of low embolic potential. Follow-up of the areas with nanoparticles vascular blockage at 24 h and 48h, revealed the persistence of this effect, only in the precapillary arterioles and capillaries that were arising from it.

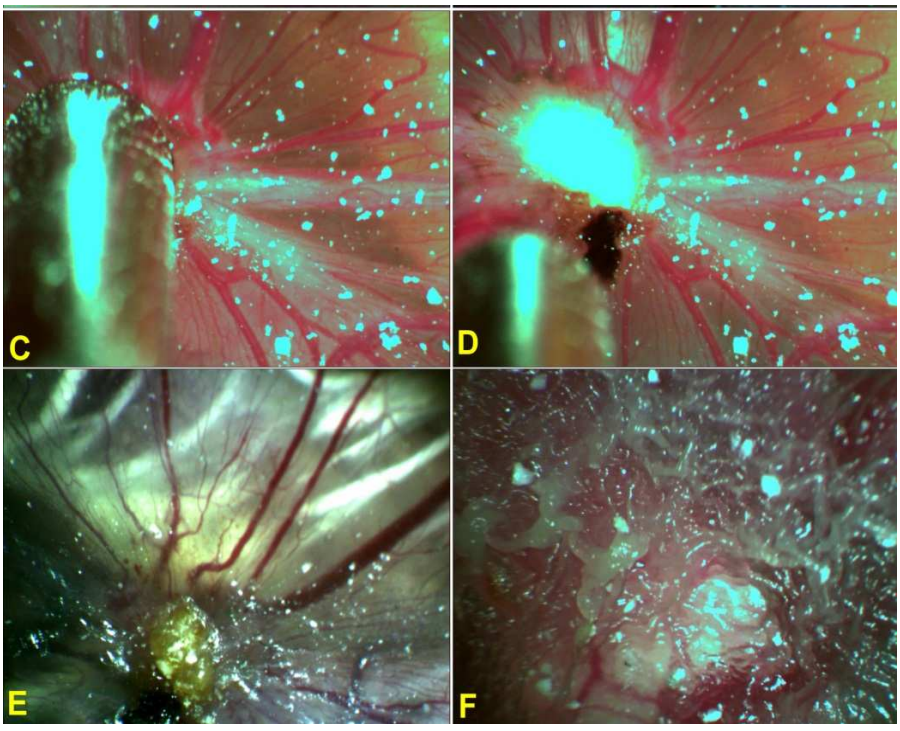
Histologic sections studied show that nanoparticles deposits localized intra- or extravascularly were not observed outside the areas exposed to the static magnetic field, which once again confirms the low embolic potential of the nanoparticle aggregates remobilized from the nanoblocked vessel.

Chapter VI. Study of targeting magnetic Fe_3O_4/salicylic acid in the tumor vessels. Testing the action of magnetic nanoparticles on tumor cells GB3B

The evaluation of the behavior of ferromagnetic nanoparticles on tumor vessels was performed on 10 chicken embryos at day 6 of development. On the chick CAM, 2 - 3 drops of standard culture medium (MEM) containing 10^5 glioblastoma cells (GB3B) per drop were implanted. After implantation, the embryos were incubated until day 11 of development, during which the xenografts developed. Seven of the embryos were injected intravenously with 0.2 mL dispersion of ferromagnetic nanoparticles 0.19%.

After injection of nanoparticles dispersion, a 0.18T NdFeB magnet was applied on the surface of tumor xenografts for 15 minutes. The eggs were returned into incubator until day 16, when tumor xenografts with the surrounding chick CAM were collected and fixed in 4% neutral formalin for 24 hours. After 120 hours from glioblastoma tumor cell implantation, multiple tumours appeared in the chick CAM surface. These stimulated angiogenesis, shown by the appearance of numerous vessels with characteristic pattern "spoke wheel" around them. There is a tendency of tumor cells to migrate along vessels around xenograft CAM thickening, which starts to become opaque at mesoscopic examination. Injection of ferromagnetic nanoparticles and application of a magnet on tumor xenografts for 15 minutes, produced a partial nanoblockage of the vessels that can be observed around the CAM of the xenograft.

In embryos injected with ferromagnetic nanoparticles, the tumors no longer developed, but continued to exert an angiogenetic effect on the vessels surrounding the chick CAM. Macroscopic analysis of the chick CAM around the nanoblocked tumor vessels does not show thickening. CAM is translucent and prominent vessels can be observed.



Stereomicroscopic appearance of glioblastoma xenografts implanted on the chick CAM.
C) applying the magnet on the tumor after intravenous injection of ferromagnetic nanoparticles; D) peritumoral vessels blocking the action of the magnetic field; E) growth appearance at 168 hours after injection;
F) xenograft tumor appearance in control group, uninjected dispersion of nanoparticles.

On the 3 embryos from the control group, which were not injected intravenously with the nanoparticles solution, the tumors continued to grow and unite to form an irregular cerebriform surface area on the chick CAM. The surrounding chick CAM is thickened, opaque and makes visualization of vessels using a stereomicroscope difficult.

In the group that was injected with nanoparticles with salicylic acid shell, these results revealed blocking of the development of the tumor xenograft and its perivascular tendency to metastasize. With this data only, it is difficult to conclude whether reduction in tumor xenograft growth is due to the direct effect of the injected nanoparticles or the decreased blood flow due to nanoblockage.

Chapter VII. In vivo study of nanoparticles action on murine malignant melanoma model

For this study we used a murine model of malignant melanoma. Malignant melanoma was chosen for two reasons: high tumour aggresion and the angiotropism that malignant melanoma cells express. This latter mechanism means that some tumor cells metastasize by moving through the blood vessel wall and has been described for the first time on human melanoma xenograft implanted on the chicken embryo CAM (25). This study had several stages: achieving murine model of murine melanoma; testing the property of metastasizing tumor cells of murine melanoma on the chick CAM *in vivo* model; oral treatment of the developed murine melanoma with ferromagnetic nanoparticles solution.

For the murine model of malignant melanoma, mice from the C57BL/6 bred were inoculated with either type B16F1 tumor cells (low metastatic potential), or B16F10 tumor cells (increased metastatic potential). For the experiment we used two female C57BL/6 for each cell line. They were injected subcutaneously with 250.000 cells/mL in a saline solution.

After 14 days from implantation, macroscopically visible tumours were observed at the site of subcutaneous injection of tumor cell suspension. The small tumors begin to grow rapidly and reach sizes around 10 mm diameter after 21 days of inoculation.

The appearance of the implantation area (black arrow) from the line B16F10 tumor cells at 21 days after implantation

The chick CAM model of murine melanoma

The experimental model of malignant melanoma on the chick CAM at 8 day of development, involved the collection of primary tumor tissue induced by B16F1 and B6F10 cells from mice through a fine aspiration puncture. Collecting tumor tissue and transplantation of tumor xenograft on chick CAM are the steps used for obtaining the transplanted murine tumor xenograft.

The daily analysis of chick CAM revealed two distinct types of B16F10 murine melanoma tumor tissue development. A locally reduced development of B16F10 tumor xenograft was associated with a lack of angiogenesis in the chick CAM vessels around the xenograft and early migration tendency of xenograft tumor cells on the chick CAM surface.

Having low metastatic potential, for B16F1 tumor cells, stimulation of angiogenesis in the chick CAM vessels around the tumor xenograftis shown by the appearance of 'spoke wheel' in chick chorioallantoic membrane vessels layout, with an increasing volume of the tumor. In this case, the tumour is visible after three days from implantation. This experiment shows that murine melanoma

xenograft grows on chicken embryo CAM and preservs the histological characteristics and primary murine tumor metastatic potential. Therefore, this model can be used to verify the metastatic characteristic of primary malignant melanoma and it is necessary to confirm the metastatic character of tumors developed in mice.

Evaluating of Fe_3O_4/salicylic acid nanoparticles on tumor cells

To investigate the potential effect of iron oxide nanoparticles with salicylic acid shell on B16F1 or B16F10 tumor cells type, C57BL/6 bred, 13 females mice, 6 months old, 23 - 29 g were used.

The animals were divided into two groups: 8 mice with B16F10 xenograft (two were controls), and 5 mice with B16F1 xenograft (one was control). All animals were injected subcutaneously in the left posterior thigh with 0.5 mL tumor suspension (250.000 cells/mL).

All animals were properly fed and grown at 22^0C and 46% humidity. The animals were monitored for tumor development evolution for 2 weeks. One death was recorded in B16F1 group, at only 24 hours after injection, probably due to tumor emboli.

Starting with the 14th day after inoculation, all mice included in the study were placed in separate cages from the mice in the control groups. Mice in the study group received MNP dispersion in their drinking water. The dose/animal was 0.5 mL MNP dispersion to 4.5 mL water/24h (26).

At the end of this study the following were observed:

- 90% of the animals developed tumors at the site of implantation;
- survival increased only in animals receiving daily nanoparticle dispersion in drinking water (0.5 mL:4.5 ml)
- occurrence of metastases was reduced in the study group.

CONCLUSIONS

1. Iron oxide nanoparticles with salicylic acid shell were synthesized using the modified Massart method; MNP structure was confirmed by specific methods: TEM microscopy, FT-IR spectroscopy, DLS, XRD, gravimetry.
2. The Fe_3O_4/salicylic acid nanoparticles synthesized were tested in subsequent studies: 60.3 nm mean diameter and 67 mV Zeta potential conferred them increased stability of the aqueous dispersion.
3. *In vivo* testing of ferromagnetic nanoparticles dispersion on chick CAM vessels demonstrated increased bioavailability after intravenous administration. Embryo survival rate in the study was over 80%, with embryo development within normal parameters.
4. The embryo had an increased capacity for retention of nanoparticles in his structures, proportional to the administrated doses. For doses exceeding the capacity of rapid retention of nanoparticles, systemic long-term persistence of over 12 hours was found.
5. Intravascular behavior of the injected doses has shown a decreased risk of embolization after enteral or parenteral administration.
6. The possibility of targeting and accumulation of ferromagnetic nanoparticles in the tumor vessels under the magnetic field action suggest the opportunity of use in biomedical field in controlled blocking of the blood flow in tumor vessels.
7. Treatment on glioblastoma cell lines and human fibroblasts indicates that low concentrations of MNP, for a period of 24, 48 and 72 hours, had no cytotoxic effect.
8. *In vivo* dispersion action of intravenous MNP on glioblastoma and breast cancer xenografts implanted on the CAM hampered the development of tumor xenografts and its perivascular metastasis. It is though difficult to say whether the blocking effect of xenograft tumor development is due to a possible direct effect of nanoparticles or reduction by nanoblockage of the blood flow in the chick CAM vessels surrounding the xenograft, responsible for supporting the viability of the xenograft.
9. Oral administration of nanoparticles dispersion in C57BL/6 mice with B16F10 and B16F1 tumor xenografts showed that treated animals had a longer life span than animals from the control groups; also, treated animals developed less metastases than untreated animals.

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