

Chick Chorioallantoic Membrane Model as a Preclinical Tool for Nanoparticles Biology Study

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Abstract

A long medical history of the chick embryo chorioallantoic membrane model (CAM) and its major advantages (e.g. high-vascularized membrane, immunodeficiency, easy handling, quick and inexpensive) explain their expanding utility in many fields like cancer research, angiogenesis, tissue morphogenesis, toxicology studies, etc. Our *in vivo* study examined the biocompatibility and the embolic potential of the aqueous dispersion of the Fe₃O₄/salicylic acid magnetic nanoparticles (MNPs) with various diameters, and also the utility of the CAM model in the nanoparticles design and toxicity evaluation. The MNPs were synthesized by modified Massart method and characterized with Dynamic Light Scattering (DLS) in order to establish their hydrodynamic diameter range (< 50, 50-100, and > 100 nm). All MNPs dispersions were intravenous administered into CAM vessels and their biological properties evaluated with/without magnetic targeting for establish the embolic risk. The results showed that the 50-100 nm diameter range MNPs had no embolic risk, and a safety intravenous administration. Tissue MNPs deposits were biocompatible with embryos and chicken. These results validate the CAM as a suitable model to provide remarkable information about real-time visualization of MNPs intravascular behavior and properties.

Keywords: CAM model, nanoparticles design, Fe₃O₄/salicylic acid nanoparticles, biocompatibility, biodistribution

1. Introduction

Aristotle reported in "Historia Animalium" his theory about the commonly fundamental characteristic between the chick embryo and human [1]. After many years of experimentation, the chick embryo CAM begins a well-known model to study cancer and cardiac biology and to understand human disease [2, 3]. The natural immunodeficiency up to fourteen days old recommends the CAM as a platform for xenograft human tissues [4, 5]. Also, the CAM is a useful model to study angiogenesis, cancer metastasis, carcinogenesis, and to visualize the tumor vascular dynamic and the tumor blood-flow, and recently it was recognized as a preclinical model [3, 6]. Moreover, Kue et al. sustains the suitability of CAM as a predictive model for testing the safety and to establish the pharmaceutical substances toxicity [7]. To understand the cancer biology and to design the new drugs or carriers the chick embryo is an

in vivo model which provides some major advantages: well-vascularized and easy to access CAM, the lack of a developed immune system, the possibility to observe in real time the morphological changes, the fast development of tumor xenografts, easy handling, quick model, high reproducibility and not expensive [1, 8]. MNPs have significant utility in medical field (imagistic, drug delivery, immunoassay, etc.) especially based on their unique magnetic properties, and *in vivo* biocompatibility [9, 10]. The favorable MNPs toxicity profile is increased by coating with biocompatible substances (lipid, protein, salicylic acid, etc.) [11]. The nanoparticles design is involved in choosing the administration mode and also in their future biomedical applications. For example a long circulation half-life of MNPs is useful for targeting tumors and *in vivo* efficient imaging [10, 12]. To enhance the MNPs bioavailability, it is necessary to improve the nanoparticle surface with different molecules (e.g. PEG, dextran, starch, etc.) that resist to the reticuloendothelial system interactions [11, 13, 14]. The aim of our study was to investigate the utility of CAM model to establish the optimal hydrodynamic diameter in order to synthesize and design MNPs without embolic risk after intravascular administration, and to study the MNPs biological properties. We used aqueous dispersions of Fe₃O₄/salicylic acid nanoparticles with different diameter range.

2. Materials and Methods

Fe₃O₄/salicylic acid nanoparticles with different diameters were synthesized by modified Massart method [9] and analyzed by DLS with Brookhaven 90 PLUS nanoparticle size analyzer in order to establish MNPs hydrodynamic diameter and Zeta potential. All measurements were performed in aqueous dispersion and were determined at an angle of 90° and 21°C. Data were given as average of three individual measurements.

2.1. CAM assay for MNPs optimal diameter determination

Fresh fertilized white Leghorn eggs were incubated at 37.5°C and 60% relative humidity. On day three of embryo development a 10 mm window was cut on the top of each egg, 4 mL of albumen was aspirated, and then resealed with adhesive tape. The eggs were incubated until day 12 when the CAM vessels were sufficiently developed to allow MNPs intravascular administration by needle injection. Doses of 0.15 mL MNPs dispersion from each sample (Table 1) were intravenously injected into CAMs (one dose for each egg), and then the eggs were replaced to incubator. After 30 minutes the viability of embryos and potential intravascular emboli were inspected by operatory microscope. If no changes were observed a 0.18 T NdFeB magnet was placed for 10 minutes on the CAM surface, and then the eggs were analyzed in order to determine the embryos viability and embolic risk of the intravenously administered MNPs. All survived embryos were replaced to the incubator.

2.2. CAM assay for MNPs biological properties study

Half of embryos injected with 60.3 nm and 79.9 nm MNPs aqueous dispersion were sacrificed after 24 hours, and autopsied to harvest embryo viscera (heart, kidney, liver, and lung). The viscera fragments were processed by standard histological techniques, included in paraffin, sectioned and stained with Prussian blue in order to evaluate the visceral MNPs deposits. The remaining embryos were incubated to hatch for morphological changes evaluation.

3. Results and Conclusions

We synthesized by co-precipitation method 8 probes of stable aqueous dispersion Fe₃O₄/salicylic acid MNPs with different hydrodynamic diameters (Table 1). These aqueous

dispersions of salicylic acid-shell iron oxide MNPs with diameters between 33 nm and 277.9 nm were tested on the chick embryos CAM vessels (Fig.1).

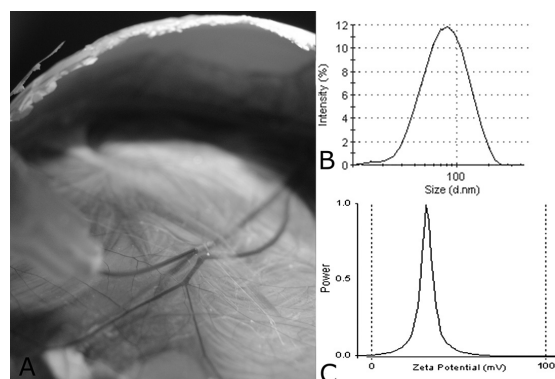


Figure 1. CAM intravenous injection (A), DLS size distribution (B), and Zeta potential value (C) of 79.9 nm Fe₃O₄/salicylic acid nanoparticles.

Table 1. Results of Fe₃O₄/salicylic acid MNPs DLS analysis

Probe	Hydrodynamic diameter (nm)	Zeta potential (mV)
1	277,9	13,24
2	209,4	41,13
3	191,3	33,41
4	79,9	35,54
5	60,3	67
6	43,5	35,83
7	41	28,97
8	33	22,43

3.1. CAM assay for optimal diameter determination of MNPs used on *in vivo* models

Preliminary *in vivo* studies conducted on the chick CAM assay were performed to choose the optimal diameter of the nanoparticles in order to respond to the following criteria: persistence in circulation for long periods of time; lack of adverse effects and complications due to the formation of intravascular emboli; option of driving targeted nanoparticles with an external static magnetic field.

The 8 synthesized probes of Fe₃O₄/salicylic acid MNPs were divided into 3 lots: lot 1 with MNPs diameter greater than 100 nm; lot 2 with MNPs diameter between 50 nm and 100 nm; and lot 3 with MNPs diameter smaller than 50 nm (Table 2).

Table 2. Fe₃O₄/salicylic acid MNPs biological properties

Lot no.	Hydrodynamic diameter (nm)	Embolic risk	Bloodstream persistency	Embryo survival rate (%)
1	277,9	+++	*	0
	209,4	++	+++	20
	191,3	+	+++	40
2	79,9	-	++	93.33
	60,3	-	++	100
	43,5	-	+	100
3	41	-	+	100
	33	-	*	100

+++ = high; ++ = moderate; + = low; - no; * = impossible to determine

3.1.1. Intravascular Lot 1 MNPs behavior

The first experiment involved testing intravascular behavior of three samples of aqueous dispersion of Fe₃O₄/salicylic acid MNPs, with their average diameter of 191.3 nm, 209.4 nm, respectively 277.9 nm on 12-day-old chicken embryos. For each sample, a dose of 0.15 mL dispersion of nanoparticles was intravenously injected in the CAM of five chicken embryos.

Right after intravenous administration of 277.9 nm MNPs dispersion, all the embryos injected formed vascular emboli that irreversible blocked the CAM capillary network, shortly followed by death of the embryo (Fig. 2 C and D).

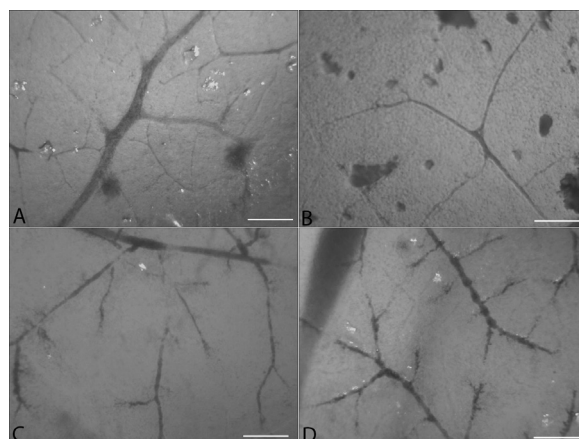


Figure 2. Morphological aspects of the chicken embryos CAM injected with 41 nm (A), 43 nm (B), 209.4 nm (C) and 191.3 nm (D) dispersion of MNPs (bar = 1mm).

No intravascular emboli were observed in the chicken CAM embryos injected with 191.3 nm or 209.4 nm MNPs dispersion. All injected embryos survived. Those embryos were replaced to incubator for a period of 30 minutes, and then a 0.18 T NdFeB magnet was placed for 10 minutes on the surface of each embryo CAM. After magnetic field removal, the nanoparticles aggregates formed into the CAM vessels have mobilized and generated vascular emboli, leading to the death of 4 embryos injected with 209, 4 nm MNPs dispersion, and of 3 embryos injected with 191.3 nm MNPs dispersion. The results of this experiment showed that the intravenously administered MNPs with a diameter greater than 190 nm have a direct embolic potential (277.9 nm MNPs dispersion) or induced by a static magnetic field (191.3 nm and 209.4 nm MNPs dispersion) with 80% embryo mortality. Therefore, the MNPs dispersion with diameter larger than 190 nm may not be used for intravascular administration on *in vivo* model experiments.

3.1.2. Intravascular Lot 2 MNP behavior

The second experiment carried out in order to test the intravenous effect of MNPs dispersion with diameters greater than 50 nm (respectively 60.3 nm and 79.9 nm). A dose of 0.15 mL of MNPs dispersion was tested on 15 chicken embryos (12-day-old) from the each dispersion. All embryos survived following intravascular injection of 60.3 nm MNPs dispersions. There was one death in the lot of embryos injected with 79.9 nm MNPs dispersion without identifying the presence of vascular emboli or a question directly related to intravascular administration of dispersion. Large intravascular MNPs aggregates were observed after 10 minutes action of a 0.18 T NdFeB magnet placed on CAM's surfaces. After removing the magnetic field the venous MNPs aggregates were redispersed by remobilization, without causing vascular emboli (Fig. 3). It also notes that the blockage of the precapillary arterioles by aggregates of nanoparticles is irreversible. This experiment revealed non embolic nature of the MNPs with size between 60 nm and 80 nm and the advantage of their safety management with a targeted static magnetic field.

3.1.3. Intravascular Lot 3 MNPs behavior

The third experiment was done to test the intravenous effect of MNPs dispersion with average diameters less than 50 nm (respectively 33 nm, 41 nm and 43.5 nm). From the each dispersion a dose of 0.15 mL was tested on five 12-day-old chicken embryos. All embryos survived following intravascular injection of MNPs dispersions. Very small intravascular

MNPs aggregates were observed after 10 minutes action of a 0.18 T NdFeB magnet placed on CAM's surfaces. These MNPs were present in the vessels only for short periods of time due to their rapid leaving blood vessel and arrest in the embryonic structures or entering through the CAM in the amniotic sac (Fig. 2 A and B). Survival rate was 100% and the embryos hatching normally. The main disadvantage of using the MNPs dispersion with diameter less than 50 nm is their short time presence in the blood vessels (around 30 minutes).

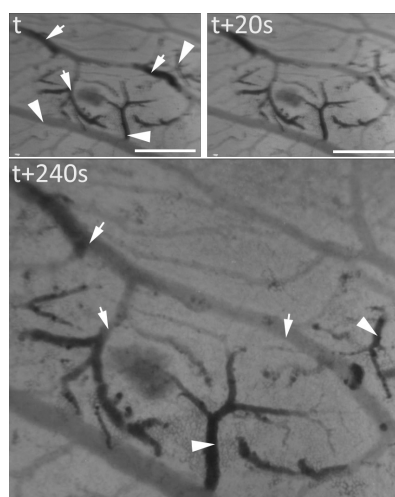


Figure 3. The chick embryo CAM injected with the 60.3 nm MNPs dispersion and magnetically accumulated into arteriolar (arrowhead) and venous (arrows) branches. Partially remobilization of venous MNPs aggregates was observed after 6 minutes (bar = 1mm).

3.2. CAM assay for MNPs biological properties study

Low embolic risk, intravascular long time persistency and good handling under the action of static magnetic field led to the selection of the Lot 2 MNPs dispersions for the biological properties evaluation on the CAM model. Five embryos from each group of Lot 2 were sacrificed 24 hours after MNPs intravenous administration to evaluate MNPs visceral deposits, and the rest of the embryos (10/9 embryos injected with 60.3 nm/79.9 nm MNPs) were incubated until hatching to evaluate the visceral MNPs deposits potential toxicity by morphological analysis of the chicken. Histological studies (Perls' Prussian blue staining) have shown MNPs deposits in the liver tissue (retained by hepatocytes and the Kupffer cells) and bloodstream (freely or retained by the monocytes). No MNPs deposits were identified in the heart, kidney, and lung tissues (Fig. 4).

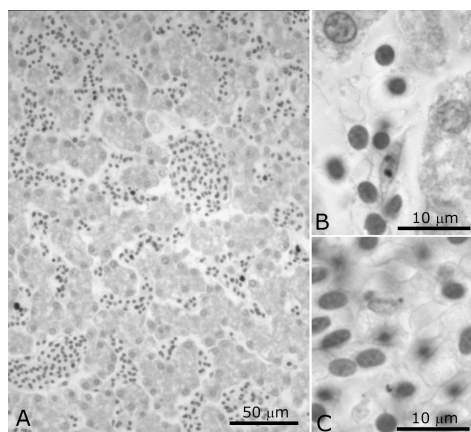


Figure 4. Fe₃O₄/salicylic acid nanoparticles deposits into Kupffer cells of the embryo liver tissue (A and B) and monocytes of the bloodstream (C) (Prussian blue staining).

All the incubated embryos hatched normally and no morphological changes were visualized to the chickens that were overseen for 7 days. Because of its easy accessibility, rich vasculature and natural immunodeficiency, the chicken embryo CAM has been extensively used in the study of cancer biology [1, 8], vascular development, vascular responses to injected drugs and drug pharmacokinetics, toxic effects and biodistribution [7, 15]. To date there are few reported studies about the biological properties evaluation of nanoparticles on the CAM model [9, 16, 17]. The CAM model is an attractive and alternative assay at the limit between preliminary cell-based, and costly and time consuming animal-based models [18, 19] that can be used without ethical restriction until day 14 of development [7]. The specific pattern of vessels facilitates the easy access and direct visualization with a stereomicroscope, and it makes the CAM a powerful tool to direct study the intravascular MNPs behavior with/without magnetic targeting, and to select the optimal diameter in order to obtained MNPs without embolic risk. In our work the CAM model allowed the rapid selection of favorable diameter range of Fe₃O₄/salicylic acid nanoparticles with good magnetically behavior, long time persistency in bloodstream and no embolic risk. *In vivo* MNPs distribution studies showed that liver is the main viscera for MNPs deposits [20, 21], and similar recorded data were highlighted by embryos viscera histological analysis. Normal development and hatching of embryos with liver MNPs deposits and normal morphological features of the chicken sustained the low MNPs cytotoxicity and showed the CAM model utility in establishing the nanomaterials biocompatibility.

4. Conclusions

The results of this study showed that the CAM model can be a useful and quick tool in nanoparticles design and characterization. Low embolic risk, intravascular long time persistency and good handling under the action of static magnetic field recommend the use of 60-80 nm Fe₃O₄/salicylic acid nanoparticles dispersions as magnetically targeting agents. Nanoparticles deposits in viscera and uninfluenced development of the injected embryos can be considered as a proof of biocompatibility and an open door for the future uses of CAM model in nanoparticles toxicological studies.

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