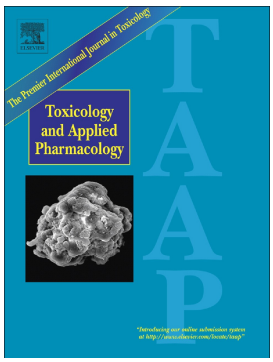

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Folic Acid Magnetic Nanotheranostics for Delivering Doxorubicin: Toxicological and Biocompatibility Studies on Zebrafish Embryo and Larvae.

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Abstract

Doxorubicin (DOXO) is a chemotherapeutic agent widely used for the treatment of solid tumors and hematologic malignancies in both adults and children. However, DOXO causes short- and long-term cardiotoxicity and others undesirable side effects, such as nephrotoxicity and neurotoxicity. Magnetic nanoparticles (MNPs) allow the delivery of drugs specifically to target place, employing an external magnet. Moreover, they may act as contrast agents in MRI providing information on the diagnostic of diverse pathologies. In this way, two functions may be combined in a unique nanosystem known as theranostic. Also, the MNPs can be modified with folic acid (MNPs@FA) to increase the uptake by cancer cells that overexpress the FA receptors. In previous works, our collaborators obtained and characterized MNPs, MNPs@FA, and MNPs@FA@DOXO. It is essential to study the biosafety of nanotheranostic, and there is no published study of Fe_3O_4 nanoparticles developmental toxicity. Because of that, this work aimed to study the *in vivo* toxicity and biocompatibility of DOXO, MNPs@FA, and MNPs@FA@DOXO using zebrafish embryo and larvae as an animal model. Viability, developmental toxicity, changes in spontaneous movement (neurotoxicity), changes in cardiac rhythm (cardiotoxicity), and efficiency of DOXO-uptake were studied. While the 48-h treatment with 50 µg/mL of DOXO resulted in a 30% larvae death and the development of significant morphological abnormalities, the treatment with MNPs@FA@DOXO and MNPs@FA did not reduce the viability and did not cause developmental abnormalities. Besides, the MNPs@FA@DOXO reduced the cardiotoxicity and promoted a more rapid and significant uptake of DOXO by zebrafish larvae.

Keywords

Doxorubicin; magnetic nanoparticles; zebrafish; biocompatibility; developmental-toxicity.

Highlights

- This work is the first studying the toxicity of Fe_3O_4 nanoparticles in zebrafish.
- Doxorubicin showed to be toxic in both zebrafish embryo and larvae.
- Folic acid magnetic nanotheranostic reduced the toxicity caused by Doxorubicin.
- Folic acid magnetic nanotheranostic proved to be a biocompatible delivery system.
- The developed nanotheranostic improved the Doxorubicin-uptake at shorter times.

Abbreviations

%DLC: percentage of drug loading capability.

%DLE: percentage of drug loading efficiency.

APTS: (3-Aminopropyl)triethoxysilane

DCC: N,N'-dicyclohexylcarbodiimide

DMSO: dimethylsulfoxide

DOXO: Doxorubicin

dpf: day post-fertilization

FA: folic acid

hpf: hour post-fertilization

hpi: hour post-incubation

MAG: magnetite

MNPs: magnetite nanoparticles

MNPs@FA: magnetite nanoparticles conjugated with folic acid

MNPs@FA@DOXO: magnetite nanoparticles conjugated with folic acid delivering doxorubicin

PBS: phosphate buffer saline

SDS: sodium dodecyl sulfate

Introduction

Doxorubicin (DOXO) is an anthracycline ring antibiotic that can inhibit the synthesis of nucleic acids (Brannon-Peppas & Blanchette, 2012), due to the DNA intercalation and the inhibition of topoisomerase II in fast-proliferating cancer cells (Jović *et al.*, 2016; Zhao & Zhang, 2017). For this reason, DOXO is one of the most widely prescribed chemotherapeutic agent (Gou *et al.*, 2011) and is used for the treatment of solid tumors (e.g., breast, ovary, gastrointestinal and small cell carcinoma of the lung) and hematologic malignancies (e.g., lymphoma and leukemia) in both adults and children (Chang *et al.*, 2014; Zhao & Zhang, 2017). However, clinical studies have shown that DOXO has many undesirable side effects, including short- and long-term cardiotoxicity (Gou *et al.*, 2011; Ibrahim *et al.*, 2009; Shafei *et al.*, 2017; Tokarska-Schlattner *et al.*, 2006; Zhao & Zhang, 2017), nephrotoxicity (Ibrahim *et al.*, 2009; Yagmurca *et al.*, 2004), and neurotoxicity (Chang *et al.*, 2014; Lopes *et al.*, 2008). Besides, others drawbacks of DOXO are non-specificity, poor solubility, poor bioavailability and early clearance from the body**.** Therefore, it is of interest to improve its activity and reduce systemic toxicity of DOXO by developing an efficient drug delivery system.

Magnetic nanoparticles (MNPs) based on iron oxides holds the promising potential to deliver therapeutic agents, employing an external magnetic field, to the desired site decreasing the deleterious side effects (Shafei *et al.*, 2017). Besides, they may act as contrast agents in MRI providing information on the diagnostic of diverse pathologies. In these regards, two functions (therapeutic and diagnostic) may be combined in a unique nanosystem known as theranostic (Azcona *et al.*, 2018).

Iron oxide nanoparticles tend to agglomerate; hence, MNPs are generally coated with surfactants or polymers to minimize aggregation (Kayal & Ramanujan, 2010). Also, the MNPs can be modified with different targeting ligands to improve the arrival to the target site and promote the internalization through receptor-mediated endocytosis. Folic acid (FA) represents an attractive option to modify MNPs because of its low cost, biocompatible, and non-immunogenic (Azcona *et al.*, 2018; Scomparin *et al.*, 2015). Besides, the folate receptors are overexpressed in several types of tumors including kidney, ovarian, brain, breast, and lung (Maeng *et al.*, 2010).

Over the last few years, zebrafish (*Danio rerio*) has frequently been employed for biosafety evaluation of nanoparticles and several anticancer nanoformulations (Igartúa *et al.*, 2015; Igartúa *et al.*, 2018; Jović *et al.*, 2016). This biosafety evaluation should include both the study of the toxicity (non-specific and undesired effects of a drug or nanoparticle) and the biocompatibility (ability to be in contact with a living system without producing an adverse effect) of drugs and nanoparticles (Vert *et al.*, 2012). Since nanomaterial studies based on cell cultures could be inconsistent and might underestimate their impacts, biosafety of nanomaterials needs to be analyzed in whole animal systems (Valdiglesias *et al.*, 2016). In this sense, zebrafish is a suitable animal model because depending on the moment of the exposure to the formulation, both developmental toxicity (embryo stage) and biocompatibility (larvae stage) can be studied. Also, zebrafish is a rapid, high-throughput, cost-effective model since they have a small size, a high fertilization rate and a rapid external development of transparent embryo (Hill *et al.*, 2005). In addition, the cardiovascular, nervous and digestive systems of zebrafish are similar to mammals (Hsu *et al.*, 2007; C. Martinez *et al.*, 2017).

In previous works, our collaborators obtained and characterized MNPs of magnetite (Fe_3O_4) and MNPs modified with FA (MNPs@FA) (Azcona *et al.*, 2018; Azcona *et al.*, 2016). More recently, they adsorbed DOXO to the MNPs@FA obtaining MNPs@FA@DOXO. Therefore, this work aimed to study the *in vivo* toxicity and biocompatibility of DOXO, MNPs@FA, and MNPs@FA@DOXO using zebrafish embryo and larvae as a high-throughput model.

Material and Methods

Materials

Ferric chloride hexahydrate (99.99%) and sodium dodecyl sulfate (SDS) were provided by Biopack (Argentina). Ferrous sulfate heptahydrate (99.99%) was provided by Mallinckrodt Chemical Works (USA). Sodium hydroxide and acetic acid (29%) were purchased from Cicarelli (Argentina). Absolute ethanol was provided by Quimicor (Argentina). (3-Aminopropyl)triethoxysilane (APTS) was provided by Avocado Research chemicals (United Kingdom). N,N'-dicyclohexylcarbodiimide (DCC)

was purchased from Fluka (Germany). Folic Acid (FA) and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (Germany). Commercial doxorubicin (DOXO) was provided by IMA Laboratories (Argentina) and donated by the Hospital Provincial of Neuquén (Neuquén, Argentina). Bidistilled water with a conductivity of about 5.00 μS was employed. All other reagents were of analytical grade and used without further purification.

Synthesis of magnetic nanosystems: MNPs, MNPs@FA, and MNPs@FA@DOXO

The magnetic core of magnetite (MAG) was prepared through inverted co-precipitation technique as our collaborators previously reported (Azcona *et al.*, 2016). A preliminary treatment with APTS was made to achieve the folic acid (FA) linked on MAG surface. This MAG-APTS obtained material was called as magnetic nanoparticles (MNPs). Subsequently, the FA was attached to MNPs throughout covalent linkage employing DCC as a coupling agent. The obtained formulation was called MNPs@FA. Our collaborators (Azcona *et al.*, 2018) have extensively studied both procedures previously. Finally, an aqueous dispersion of MNPs@FA was mixed with 2 mg/mL doxorubicin solution (DOXO) in a DOXO/MNPs@FA mass ratio of 1/10, under magnetic stirring. After 24 h of stirring in darkness, the final sample was decanted using an Nd magnet and dried in an oven at 34°C under vacuum. The formulation achieved by using this simple adsorption method was called MNPs@FA@DOXO (**Figure 1**).

Figure 1. Scheme of magnetic nanoformulations synthesis. The magnetic core was treated with APTS to obtain the MAG-APTS material (MNPs). Subsequently, the FA was attached to MNPs through covalent linkage to obtain MNPs@FA. Finally, DOXO was adsorbed into MNPs@FA surface obtaining MNPs@FA@DOXO.

Physicochemical characterization of magnetic formulations

Transmission electron microscopy (TEM, JEOL 100 CX II, Tokyo, Japan) was used to study the morphology of the MNPs@FA@DOXO. Hence, the sample was dispersed in bidistilled water, placed on 200 mesh Cu grids, and dried at room temperature.

Hydrodynamic diameter (nm) and Z potential (mV) measurements were performed in an Analytical Malvern Zetasizer® (United Kingdom) equipment using aqueous dispersions of MNPs@FA and MNPs@FA@DOXO at a 0.4 mg/mL concentration of nanoparticles and pH=7.4. All the samples were ultrasonicated for 60 min previously to the assay, and the measurements were performed at 25°C. The informed values were an average of about three repeated measurements.

Atomic absorption spectroscopy using a GBC Avanta 932 (Australia) was implemented to assess the composition of MNPs@FA and MNPs@FA@DOXO regarding total iron content. For these measurements, 10 mg of different magnetic formulations were dissolved in 25 mL of HCl 10% m/v.

UV-visible spectroscopic measurements at 480 nm were performed on a Shimadzu 160 spectrophotometer (Tokyo, Japan) to determine the amount of DOXO incorporated in MNPs@FA (Jaimes-Aguirre *et al.*, 2017). A linear calibration curve (R^2=0.9993) was constructed relating absorbance and DOXO concentrations ranging between 20 and 70 µg/mL. The drug loading efficiency (% DLE) and drug loading capability (% DLC) of the nanocarrier were calculated as follows:

$$\%\,\text{DLE} = (W1/W3) \times 100$$

$$\%\,\text{DLC} = (W1/W2) \times 100$$

W1 represents the total amount of drug in the MNPs@FA, W2 is the total mass of MNPs@FA, and W3 indicates the total initial mass of DOXO.

Study of iron release from MNPs@FA, and MNPs@FA@DOXO

The stability of the formulations was studied regarding the retention of iron. For this, two different batches containing 6.6 mg of MNPs@FA or 7.3 mg of MNPs@FA@DOXO were incubated in 10 mL of buffer phosphate saline (PBS 10 mM, pH=7.4) during 48 h at 28°C. Samples of 2 mL were withdrawn at prefixed times (2, 3, 4, 5, 6, 24 and 48 h) and filtrated to obtain a clean solution to analyze the iron content by atomic absorption spectroscopy. In all the cases, the volume was replaced with fresh PBS.

In vivo toxicity and biocompatibility: Zebrafish embryo and larvae

Animals

Adult zebrafish (*Danio rerio*) were maintained at 28.0 ± 1.0 °C in aquaria with a 14 h light − 10 h dark cycle, as we described previously (Igartúa *et al.*, 2018; C. S. Martinez *et al.*, 2018). Fishes were fed with dry flakes (TetraMin PRO®) three times a day and nauplius larvae of *Artemia* once a day. The water in the aquarium was aerated and maintained at pH 7.0 - 8.0. In this study, embryos refer to zebrafish before hatching (0-3 day post-fertilization - dpf), while larvae refer to post-hatch animals (over 3 dpf) (**Figure 2**). Embryos were obtained from natural mating and were reared in E3 medium (NaCl 5 mM, KCl 0.17 mM, $CaCl_2$ 0.33 mM and $MgSO_4$ 0.33 mM in deionized water and 50 ppb methylene blue as fungicide). Selected fertilized eggs in good condition were used for further treatment. The characteristics of eggs were determined with a Nikon SMZ800 stereomicroscope (USA).

Figure 2. Scheme of zebrafish development and *in vivo* toxicity and biocompatibility assays. In our study, embryos refer to zebrafish before hatching (0-3 day post-fertilization - dpf), while larvae refer to post-hatch animals (over 3 dpf). Embryos were obtained by natural mating; then, they were collected and selected at 1 hour post-fertilization (hpf). For embryos treatment, one non-hatched embryo at 1 hpf was placed in each well of a 96-well plate containing E3 medium. At 4 hpf, the medium was replaced by different solutions of DOXO, MNPs@FA@DOXO, or MNPs@FA. At 24 and 48 hpf, the developmental toxicity was studied. The hatching rate was analyzed until 72 hpf. For larvae treatment, three non-hatched zebrafish embryos at 1 dpf were placed in each well of a 96-well plate containing E3 medium and incubated for additional 4 days. At 5 dpf, the medium was replaced by different solutions of DOXO, MNPs@FA@DOXO, or MNPs@FA. At 4, 24, and 48 hour post-incubation (hpi), the viability, the cardiotoxicity and the morphological changes were analyzed. At 1, 4, 24, and 48 hpi the spontaneous movement (neurotoxicity) was studied. Also, at 48 hpi the DOXO-uptake was analyzed by optical and fluorescence microscopy.

Compliance with ethical standards

All zebrafish procedures were performed in strict accordance with the National Institute of Health guidelines for animal care and maintenance. The study protocols were approved by the Institutional Animal Care Committee of the National University of Quilmes (CE-UNQ 2/2014) (Buenos Aires, Argentina) and Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) (CICUAL-UNQ 013-15 and 014-15).

Zebrafish embryo: Treatment

For the zebrafish embryo´s treatment, one non-hatched embryo at 1 hour post-fertilization (hpf) was transferred to each well of a 96-well plate containing E3 medium and incubated at 28 °C. At 4 hpf, the medium was replaced by 250 µL of two-fold-serial dilutions prepared in E3 medium of DOXO (3.12-50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA), or MNPs@FA (70.75-1132 µg/mL).

Zebrafish embryos: Hatching and viability

Embryonic development was monitored from 4 to 52 hpf using a stereomicroscope. Hatching and viability were determined at different time points (24, 28, 33, 48, and 52 hpf) and defined as a percentage of live/hatched embryos respect to the total of embryos per treatment (n=32) (C. S. Martinez *et al.*, 2018). Besides, at 8, 24 and 48 hpf, different endpoints were observed. Lethal endpoints included coagulation and absence of the heartbeat, whereas sublethal endpoints included malformation of head, eyes, tail, heart, bent spine, deformity of yolk, reduction of pigmentation and delay or reduction of hatching (adaptation from (Girardi *et al.*, 2017)). Embryos were defined as normal if none of the sublethal endpoints were observed; malformed if one or more sublethal endpoints were observed; or deceased if a lethal endpoint was detected.

Zebrafish larvae: Treatment

For the treatment, three non-hatched zebrafish embryos at 1 dpf were placed in each well of a 96-well plate containing E3 medium and incubated for an additional of 4 days at 28°C. At 5 dpf, the medium was replaced by 250 µL of two-fold-serial dilutions prepared in E3 medium of DOXO (3.12-

50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA) or MNPs@FA (70.75-1132 µg/mL).

Zebrafish larvae: Viability

Viability was studied at 4, 24, and 48 hour post-incubation (hpi) with a stereomicroscope. It was considered that the larvae were dead when no heartbeat was observed. Viability was expressed as a percentage of the live larvae respect to the total of larvae per treatment (n=72).

Zebrafish larvae: Neurotoxicity

The spontaneous movement was studied in a multichannel analog to digital converter system (WMicrotracker, Designplus SRL, Argentina) as we previously described (Igartúa *et al.*, 2015; Igartúa *et al.*, 2018). Activity events were recorded for 15 min at 1, 4, 24, and 48 hpi, at room temperature. The spontaneous movement was expressed as the percentage of the movement in the non-treated larvae control. Changes in spontaneous locomotor activity events could reflect the neurotoxicity of the different treatments (Prieto *et al.*, 2012; Selderslaghs *et al.*, 2013), as well as a morphological or lethal effect. For each assay, eight technical replicates and three biological replicates were used for each dilution (n=24).

Zebrafish larvae: Cardiotoxicity

The heart rate of zebrafish was assessed at 48 hpi. Control and experimental zebrafish larvae were individually transferred to a slide with sodium carboxymethylcellulose and placed under a trinocular stereomicroscope. The heart rate was determined by counting the number of beats every 15 s and expressed as beats per minute. The results were expressed as a percentage with respect to the heart rate in the non-treated larvae control. Experiments were performed two times on nine larvae per group (n=18). The difference between the heart rates of control and treated larvae was regarded as cardiotoxicity (Berghmans *et al.*, 2008).

Zebrafish larvae: Morphological Changes

The larvae were photographed with a Microsoft camera at 4, 24 and 48 hpi to determine possible morphological changes. Several morphological alterations as bent spine, jaw malformation, opaque head region, small head, opaque liver, opaque yolk salc, yolk not depleted, uninflated swim bladder, edema and tail malformation were observed. Fish were scored based on the degree of morphological anomalies [0=no visible toxic effects; 1=minor, one to two morphological anomalies; 2=moderate, three to four effects; 3=severe, more than four minor toxic effects; and 4=dead] (Adaptation from (Yang *et al.*, 2014)). The score of individual larvae determined the mean toxicity score for each. Nine technical replicates and two biological replicates were used for this assay (n=18).

Zebrafish larvae: *In vivo* formulation-uptake

With the aim of study the *in vivo* DOXO-uptake, zebrafish larvae were treated with all concentrations of DOXO, MNPs@FA@DOXO or MNPs@FA and were photographed 4, 24, and 48 hpi in an optical stereomicroscope (n=18). DOXO can be seen as a red coloration in the digestion system of larvae. Besides, to quantify the DOXO-uptake, zebrafish larvae were exposed to 25.0 µg/mL of free or encapsulate DOXO and were individually transferred at 48 hpi to a slide and placed under a Cytation 5 fluorescence microscopy (USA). The larvae were excited at 589 nm, and the intensity emission at 647 nm (red fluorescence) was quantified with the image-J program. Experiments were performed two times on nine larvae per group (n=18).

Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Graph Pad Prism v6.0 software. ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test or by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-tests were used depending on the obtained data. Differences were considered to be significant only when $p<0.05$. The different statistical tests used are detailed within the figures´ captions.

Results and Discussion

Characterization of magnetic nanoformulations

Our collaborators obtained and characterized raw magnetite nanoparticles (MNPs) and MNPs modified with folic acid (MNPs@FA) (Azcona *et al.*, 2018; Azcona *et al.*, 2016). More recently, they adsorbed doxorubicin (DOXO) to the MNPs@FA obtaining MNPs@FA@DOXO. This adsorption aimed to confer the theranostic character to the magnetic nanosystem. Each obtained batch was physicochemically characterized to verify the reproducibility of the drug adsorption process. Transmission electron microscopy was performed to study the morphology and aggregation state of MNPs@FA@DOXO **(Figure 3 a)**. Spherical nanoparticles, aggregated in a matrix formed by the excipients present in the commercial formulation (lactose and mannitol), are observed. Also, hydrodynamic diameter and Z potential measurements were performed to evaluate the influence of the drug in such properties **(Figure 3 b and c)**. The MNPs@FA presented a hydrodynamic diameter of 453.7±10.61 nm, while the MNPs@FA@DOXO had a diameter of 597.5±34.82 nm. This significant increase ($p<0.01$) in size may be considered as an evidence of DOXO adsorption on the MNPs@FA surface. Regards to the surface charge of nanoformulations, MNPs@FA presented a Z-value of -33.07±0.208 mV, meanwhile MNPs@FA@DOXO had a Z-value of -30.77±0.208 mV. The slight variation in the Z potential value may be ascribed to the way in that DOXO linked to the MNPs surface, and it is a commonly observed in MNPs systems (Montiel Schneider *et al.*, 2018). The loading efficiency and loading charge capability of MNPs (%DLE and %DLC) were achieved by UV-Vis measurements, reaching 37.2% and 4% respectively. These values are suitable for the potential therapeutic action of nanosystems considering the data reported in the literature for *in vivo* studies (Augustin *et al.*, 2016; Luong *et al.*, 2017; Mosafer *et al.*, 2017).

Iron plays a critical physiological role in important organic metabolic pathways due to its capacity to switch between ferric (Fe^{3+}) and ferrous (Fe^{2+}) ionic forms by readily accepting and donating electrons. Nevertheless, iron levels in the organism are strictly controlled because an excess of this metal can be very toxic (Valdiglesias *et al.*, 2016). Iron leaching from MNPs needs to be studied because of an increase in the iron levels, and pathological conditions (such as cancer, arthritis or

hypertension) could lead to increased oxidative damage and toxicity. Because of these facts, the stability of prepared MNPs was assessed by incubating them in aqueous media simulating the zebrafish incubation conditions. Then, the composition regarding total iron content was measured by atomic absorption spectroscopy **(Figure 3 d).** Also, the cumulative release of iron from nanoparticles formulations was studied with the objective of predicts the toxic effects that these formulations could present on zebrafish **(Figure 3 e)**. The MNPs@FA presented 24.73 mg of total iron per 100 mg of formulation and released 3.6% of this iron after 48 hours of incubation. On the other side, MNPs@FA@DOXO was composed of 20.78 mg of iron per 100 mg of formulation, releasing 2.8% in 48 hours. There is a significant difference in the total iron release between both nanoparticles, even though it is low, that could be considered for the analysis of the toxicity tests in zebrafish.

Figure 3. *In vitro* characterization of magnetite nanoparticles. (a) Transmission electron microscopy of MNPs@FA@DOXO. **(b)** Hydrodynamic diameters of MNPs@FA and MNPS@FA@DOXO. **(c)** Z-potential of MNPs@FA and MNPS@FA@DOXO. **(d)** Total iron content per 100 mg of MNPs@FA or MNPs@FA@DOXO. **(e)** Total iron release after 48 h stirring from MNPs@FA or MNPs@FA@DOXO. Statistics were performed by unpaired t-test (*p<0.05; **p<0.01; ***p<0.001).

In vivo toxicity: Zebrafish embryos

Zebrafish (*Danio rerio*) were frequently employed for biosafety evaluation of nanoparticles because dependent on the moment of the formulation exposure to the both developmental toxicity and biocompatibility can be studied. At 5-day post-fertilization (dpf) the brain, heart, liver, pancreas, kidneys, intestine and sensory systems of larvae are fully functional (de Esch *et al.*, 2012). Therefore, an administration before 5 dpf could be used to study the effects in the formation of organs (developmental toxicity); otherwise, an administration on 5 dpf or later allowed to study the effect on already formed and functional organs (biocompatibility). In this work, we used zebrafish embryo (0-3 dpf) to analyze the developmental toxicity of DOXO, MNPs@FA, and

MNPs@FA@DOXO, and zebrafish larvae (5-7 dpf) to study the biocompatibility of the same formulations. Little information exists on the toxic effects of magnetite nanoparticles, and to the best of our knowledge, there is no published study on developmental toxicity caused by Fe_3O_4 nanoparticles in zebrafish, highlighting the novelty of this work.

This section will cover the selection of the concentrations that we used to study the effects on zebrafish. The commercial injectable solution of DOXO is 2000 µg/mL. However, according to Jovic *et al.* 2016, the plasma kinetics of DOXO exhibits an initial half-life of approximately 8 min followed by a terminal half-life of about 30 h; and the major exposure to organs occurs during the terminal phase where DOXO concentrations are less than 0.054 µg/mL (Jović *et al.*, 2016). With the aim of evaluating intermediate concentrations, we used doses between 3.12 and 50.0 µg/mL of DOXO. On the other hand, according to Kayal & Ramanujan 2010, iron oxide nanoparticles are commercially sold as MRI contrast enhancement agents (FDA approved) and are biocompatible in the doses required for therapeutic use (Kayal & Ramanujan, 2010). The human clinical dose of iron nanoparticles is around 45 µmol of total iron/kg (Corot *et al.*, 2006). Mainly, the clinical dose for AMI-121 contrast agent is between 84-220 µg of iron/mL and for OMP is 500 µg of iron/mL (Wang *et al.*, 2001). In this work, we used concentrations equivalent to 20 up to 280 µg of iron/mL, which correspond to 70.75-1132 µg/mL of MNPs@FA. In the case of MNPs@FA@DOXO, a concentration range between 3.12 µg/mL of DOXO in 70.75 µg/mL of MNPs@FA and 50.0 µg/mL of DOXO in 1132 µg/mL of MNPs@FA was used.

First, the developmental toxicity of DOXO, MNPs@FA@DOXO, and MNPs@FA on zebrafish embryos was studied. For this, embryos of 4 hour post-fertilization (hpf) were exposed to the formulations and the effects on the viability **(Figure 4),** the hatching rate **(Figure 5**) and the morphological changes **(Figure 6 and Figures S1, S2, and S3)** were analyzed.

Figure 4. Viability of zebrafish embryos exposed to DOXO, MNPs@FA@DOXO or MNPs@FA. Zebrafish embryos of 4 hpf were exposed to different concentrations of DOXO (3.12-50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA), or MNPs@FA (70.75-1132 µg/mL). Viability was studied at 24 and 48 hpf. Viability is expressed as the percentage of the live embryo respect to the total

of embryos, and data are shown as mean ± SD. Statistics were performed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. No significant differences respect to the non-treated larvae control were obtained.

None of the formulations in the concentrations tested significantly reduced embryo viability up to 48 hpf respect to the non-treated embryo control. Despite not producing lethal endpoints, the formulations presented different effects on the hatching and the development of these embryos. The treatment with 50.0 µg/mL of free DOXO or in MNPs@FA@DOXO significantly reduced the number of embryos hatched compared to the control, both at 33 hpf and 52 hpf. This result is consistent with that obtained by Chang *et al.* (2014), who observed that the hatching rates of the 25.0 and 50.0 µg/mL DOXO groups were low and showed embryonic developmental delay and highly significant toxicity (Chang *et al.*, 2014).

On the other hand, the exposure to 3.12 µg/mL and 12.5 µg/mL of MNPs@FA@DOXO produced an increase in hatching with respect to the control at 33 hpf. This increase in hatching mediated by the MNPs@FA@DOXO was also observed in the MNPs@FA, where at 33 hpf there was a concentration-dependent effect. The increase in hatching rate at 33 hpf mediated by the nanoparticles could be due to a physical effect by covering the corium and weakening it, or else to a physiological effect. However, this last option was discarded because there were no morphological changes in development. At 52 hpf, no significant changes in hatching rate were obtained for MNPs@FA treatment in any of the concentrations tested with respect to the non-treated control.

Figure 5. Hatching rate of zebrafish embryos exposed to DOXO, MNPs@FA@DOXO or MNPs@FA. Embryos of 4 hpf were exposed to different concentrations of DOXO (3.12-50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA), or MNPs@FA (70.75-1132 µg/mL). **(a)** Hatching rate was studied at 24, 28, 33, 48, and 53 hpf. Hatching is expressed as the percentage of the hatched embryo respect to the total live embryo, and data are shown as mean ± SD. **(b)** Hatching of treated embryo presents significant differences with respect to the control non-treated embryo at 33 and 52 hpf. Statistics were performed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

To study the effects on the development, embryos were treated from 4 to 52 hpf (48 hpi) and then photographed. In **Figure 6**, the effects of the concentrated samples are presented as a comparison; while in the **Figures S1, S2 and S3** photographs of all the concentrations tested are shown. In addition to the effects on hatching rate, it was observed that embryos treated with 12.5, 25.0 and 50.0 µg/mL of free DOXO presented severe morphological abnormalities, including cardiac edema and bent spine, as well as an altered cardiac rhythm (**See video in the supplementary material**). This result is consistent with those obtained by Jovic *et al.* (2016) who found that, at doses higher than 0.5 µg/mL of DOXO, zebrafish embryo presented disturbed cardiovascular functions; most of them exhibited pericardial edemas upon 25 and 50 µg/mL of DOXO (Jović *et al.*, 2016). Also, Raghavan *et al.* (2016) found that embryos treated with 25 µg/mL of DOXO for a period of 96 hpf showed deformities like bent tail and yolk sac edema (Raghavan *et al.*, 2016).

The MNPs@FA did not cause morphological abnormalities in any of the concentration tested. On the other hand, the embryos treated with 50.0 µg/mL of MNPs@FA@DOXO presented developmental morphological changes. The embryos treated with 12.5 and 25.0 µg/mL of MNPs@FA@DOXO did not present significant morphological abnormalities. These results are an indicator that the absorption of the DOXO in the nanoparticles to obtain the MNPs@FA@DOXO reduces the developmental toxicity of the drug. This reduction in toxicity is not because the nanoparticle reduces the uptake of the drug to the embryo, since as can be seen in **Figure 6**, both the embryos treated with the free DOXO and MNPs@FA@DOXO showed the yolk with red coloration.

Zhu *et al.* (2012) reported that 50 and 100 µg/mL of hematite (Fe_2O_3) magnetic nanoparticles administered to zebrafish at 4 hpf caused the dead of 45% and 75% of embryos, respectively, showing time and dose-dependent development toxicity of Fe_2O_3 (Zhu *et al.*, 2012). Also, they demonstrated that doses higher than 10 µg/mL of hematite nanoparticle displayed significant embryo-hatching delay and toxicity. These results are opposite to those obtained in this work. The difference could be due to our MNP had FA attached to the surface, making the nanoparticle biocompatible and less toxic.

Figure 6. Morphological abnormalities of zebrafish embryo exposed to DOXO, MNPs@FA@DOXO, or MNPs@FA. Zebrafish larvae of 4 hpf were exposed to DOXO (25.0-50.0 µg/mL), MNPs@FA@DOXO (25.0-50.0 µg/mL of DOXO in 566.0-1132 µg/mL of MNPs@FA) or MNPs@FA (566.0-1132 µg/mL). Sublethal morphological changes as heart edema and bent spine were observed in DOXO treated embryos at 52 hpf (48 hpi).

In vivo biocompatibility: Zebrafish larvae

Additionally to the assays performed on zebrafish embryo, zebrafish larvae of 5 dpf were exposed to DOXO, MNPs@FA@DOXO, and MNPs@FA to study the biocompatibility of the novel formulations. In the first place, viability was studied at 4, 24 and 48 hour post-incubation (hpi) **(Figure 7).** None of the treatments reduced the larvae-viability, except the treatment with 50.0 µg/mL of DOXO, which produced significant mortality of 27% of the larvae at 48 hpi. The administration of 50.0 µg/mL of MNPs@FA@DOXO did not reduce the viability, showing reduced toxicity.

Figure 7. Viability of zebrafish larvae exposed to DOXO, MNPs@FA@DOXO or MNPs@FA. Zebrafish larvae of 5 dpf were exposed to different concentrations of DOXO (3.12-50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA) or MNPs@FA (70.75-1132 µg/mL). Viability was studied at 4, 24 and 48 hours post-incubation (hpi). Viability is expressed as the percentage of live larvae respect to the total of larvae, and data are shown as mean ± SD. Statistics were performed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test. Significant differences respect to non-treated larvae control were obtained only in 50.0 µg/mL DOXO-treatment (*$p<0.05$).

In the second place, the spontaneous movement of larvae exposed to the treatments was studied at 1, 4, 24 and 48 hpi **(Figure 8)**. Measurement of the locomotor activity allows the prediction of neurotoxic effects in zebrafish larvae. Increased or reduced spontaneous movement respect to the control could be indicative of adverse effects on one or more components of the complex neuronal network that governs the early locomotor system or could be due to a morphological or lethal effect.

The treatment with 3.12 µg/mL of DOXO did not produce significant changes in locomotor activity with respect to the non-treated control. The treatment with 6.25 µg/mL of DOXO produced significant hyperactivity at 48 hpi, while 12.5 and 25.0 µg/mL of DOXO increased the spontaneous movement since 24 hpi. On the other hand, the treatment with 50.0 µg/mL of DOXO significantly increased the locomotor activity at 4 hpi, but no at 24 and 48 hpi. Findings can be explained since at 24 and 48 hpi some larvae incubated with this concentration were hyperactive while others began to die (since lethality was previously observed) and give lower average movements. According to Tacar *et al.* (2013) the neurotoxicity caused by DOXO is indirect because this drug is unable to cross the blood-brain barrier (DOXO stimulates the production of TNFα and consequent production of inflammatory cytokines by microglia cells) (Tacar *et al.*, 2013). However, zebrafish do not have fully functional blood-brain barrier until 10 dpf, so in this animal model, the DOXO could be generating effects directly on the larval brain.

In the case of MNPs@FA@DOXO, lower concentrations (3.12, 6.25 and 12.5 µg/mL) produced significant hyperactivity in a shorter time (1 and 4 hpi) with respect to free DOXO. This result could be interpreted as an increase in the arrival of DOXO to the brain, mediated by MNPs@FA, which produces a faster effect at lower concentrations than for the non-delivered DOXO. The MNPs@FA did not produce significant changes with respect to the control at any time in concentrations from 70.75 to 566.0 µg/mL.

Figure 8. Neurotoxicity of DOXO, MNPs@FA@DOXO, and MNPs@FA studied as changes in spontaneous movement on zebrafish larvae. Zebrafish larvae of 5 dpf were exposed to DOXO (3.12-50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA) or MNPs@FA (70.75-1132 µg/mL). The spontaneous movement was measured at 1, 4, 24 and 48 hpi and was expressed as a percentage respect to the spontaneous movement in non-treated control larvae. Values are shown as mean ± SD. Significant differences respect to non-treated control were analyzed by TWO-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test(*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

In the third place, the effects on the heart rate were studied at 48 hpi **(Figure 9).** According to Tacar *et al.* (2013), DOXO is responsible for structural alterations in cardiomyocytes of the heart, lengthening them, and producing cardiotoxicity effects (Tacar *et al.*, 2013). The treatment with 3.12 and 6.25 µg/mL of DOXO significantly increased the heart rate of zebrafish larvae, showing a cardiotoxicity effect. Higher concentrations of the free drug did not produce noticeable changes in heart rate but produced neurotoxicity effects as was previously showed. Contrary to these results, only the treatment with 25.0 and 50.0 µg/mL of MNPs@FA@DOXO increased the heartbeat. Finally, the MNPs@FA did not produce cardiac alterations in any of the tested concentrations, showing the biocompatibility of this nanoformulation.

Figure 9. Cardiotoxicity of DOXO, MNPs@FA@DOXO, and MNPs@FA tested as changes in heart rate of zebrafish larvae. Zebrafish larvae of 5 dpf were exposed to DOXO (3.12-50.0 µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA) or MNPs@FA (70.75-1132 µg/mL). Heart rate was measured at 48 hpi and was expressed as the percentage of heartbeat in treated larvae respect to the heartbeat in the non-treated larvae control. Values are shown as mean ± SD. Significant differences respect to control were analyzed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*$p<0.05$; **$p<0.01$).

Morphological changes after 48 h-treatment with DOXO, MNPs@FA@DOXO, and MNPs@FA was studied **(Figures S4, S5, and S6).** Several morphological alterations as bent spine, jaw malformation, opaque liver, yolk not depleted and uninflated swim bladder were observed in larvae treated with DOXO, but not in those treated with MNPs@FA@DOXO and MNPs@FA. Similar results were obtained when larvae were scored based on the degree of damage **(Figure 10).** The treatment with 50.0 µg/mL of DOXO produced significant morphological abnormities respect to non-treated larvae control. Neither the MNPs@FA nor the MNPs@FA@DOXO produced significant morphological changes at any of the concentrations tested.

Figure 10. Morphological abnormalities of zebrafish larvae exposed to DOXO, MNPs@FA@DOXO, or MNPs@FA. Zebrafish larvae of 5 dpf were exposed to different concentrations of free DOXO (3.12-50.0

µg/mL), MNPs@FA@DOXO (3.12-50.0 µg/mL of DOXO in 70.75-1132 µg/mL of MNPs@FA) or MNPs@FA (70.75-1132 µg/mL). Morphological changes were scored based on the degree of abnormalities of larvae at 48 hpi. Values are shown as mean ± SD. Statistical analysis was performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (****p<0.0001).

In parallel to the morphological changes, the uptake of the DOXO to the larvae by optical **(Figure 11)** and fluorescence microscopy **(Figure 12)** was studied. By optical microscopy, was observed that MNPs@FA@DOXO improved the uptake of the drug in shorter times; at 4 hpi, larvae treated with 25.0 µg/mL of MNPs@FA@DOXO had red coloration in the digestive system while 25.0 µg/mL DOXO do not cause this effect. Also, the growth retardation of larvae treated with 25.0 µg/mL DOXO can be evidenced by their less consumed yolk at 24 hpi. At 48 hpi, an important uptake of DOXO was observed in both free DOXO and MNPs@FA@DOXO treatments. To differentiate and quantify this uptake, fluorescence microscopy was performed and the emission in the yolk was quantified. The MNPs@FA@DOXO presented a significant emission with respect to the DOXO and the MNPs@FA (used as an auto-fluorescence control). Our results partially agree with those obtained by Yao *et al.* (2017), since they observed DOXO fluorescence in the eye, intestine, and liver of 6-dpf zebrafish larvae, while in this work eye-fluorescence was not observed (Yao *et al.*, 2017).

Figure 11. *In vivo* formulation-uptake studied in zebrafish larvae by optical microscopy. Zebrafish larvae of 5 dpf were exposed to DOXO (25 µg/mL), MNPs@FA@DOXO (25 µg/mL of DOXO in 566.0 µg/mL of MNPs@FA) or MNPs@FA (566.0 µg/mL). At 4, 24 and 48 hpi, the formulation-uptake was studied. DOXO can be seen as a red coloration in the digestive system of larvae. Scale bars: 500 µm.

Figure 12. *In vivo* formulation-uptake studied in zebrafish larvae by fluorescence microscopy. (a) Zebrafish larvae of 5 dpf were exposed to DOXO (25.0 µg/mL), MNPs@FA@DOXO (25.0 µg/mL of DOXO in 566.0 µg/mL of MNPs@FA) or MNPs@FA (566.0 µg/mL). At 48 hpi, the larvae were fixed, and fluorescence microscopy was used to analyze the DOXO-uptake. DOXO can be seen as a red fluorescence in the digestive system of larvae. **(b)** Quantification of red-fluorescence intensity in the digestive system of larvae was

performed with the image-J program. Values are shown as mean ± SD. Statistical analysis was performed by ONE-WAY ANOVA test followed by Dunnett´s multiple comparisons post-test (*p<0.05; **p<0.01; ***p<0.001). Scale bars: 1000 µm.

Conclusions and Perspectives

In the search for new methods of diagnosis for cancer, MNPs@FA were designed. For the best of our knowledge, little information exists on the toxic effects of magnetite nanoparticles, and there is no published study on developmental toxicity caused by Fe_3O_4 nanoparticles in zebrafish. Besides, the current cancer treatment with DOXO has many undesired effects, being the DOXO-induced cardiotoxicity the major problem. Therefore, the incorporation of this drug in a delivery system could improve the current treatment. Thinking about these points, the nanotheranostic MNPs@FA@DOXO were development. In this context, it recognizes the importance of studying the toxicity and biocompatibility both of the MNPs@FA and the MNPs@FA@DOXO.

DOXO showed to be toxic in both zebrafish embryo and larvae in a concentration-dependent way. The 48-h treatment with 50 µg/mL of DOXO resulted in a 30% larvae death and the development of significant morphological abnormalities. In contrast, none of the MNPs in the concentrations tested significantly reduced embryo and larvae viability with respect to the non-treated control up to 48 h of treatment. Despite not producing lethal endpoints, the MNPs@FA@DOXO presented different effects on the hatching and the development of these embryos and larvae, in both stages due to the presence of DOXO. However, the MNPs@FA@DOXO reduced the cardiotoxicity caused by free DOXO and promoted a significant uptake of DOXO at shorter times by zebrafish larvae.

For all the above mentioned, we can conclude that a biocompatible system for the delivery of DOXO was developed. This formulation could be implemented for the treatment and diagnosis of cancer. In that sense, trials in higher animals should be carried out. Also, further experiments in zebrafish should be performed with the objective of understanding the metabolization process of the drug and the novel nanomaterial. This work constitutes the first steps in the study of magnetite nanoparticles in zebrafish and sets the precedents for the optimization of nanotheranostic.

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Declaration of interest

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Highlights

- This work is the first studying the toxicity of Fe_3O_4 nanoparticles in zebrafish.
- Doxorubicin showed to be toxic in both zebrafish embryo and larvae.
- Folic acid magnetic nanotheranostic reduced the toxicity caused by Doxorubicin.
- Folic acid magnetic nanotheranostic proved to be a biocompatible delivery system.
- The developed nanotheranostic improved the Doxorubicin-uptake at shorter times.

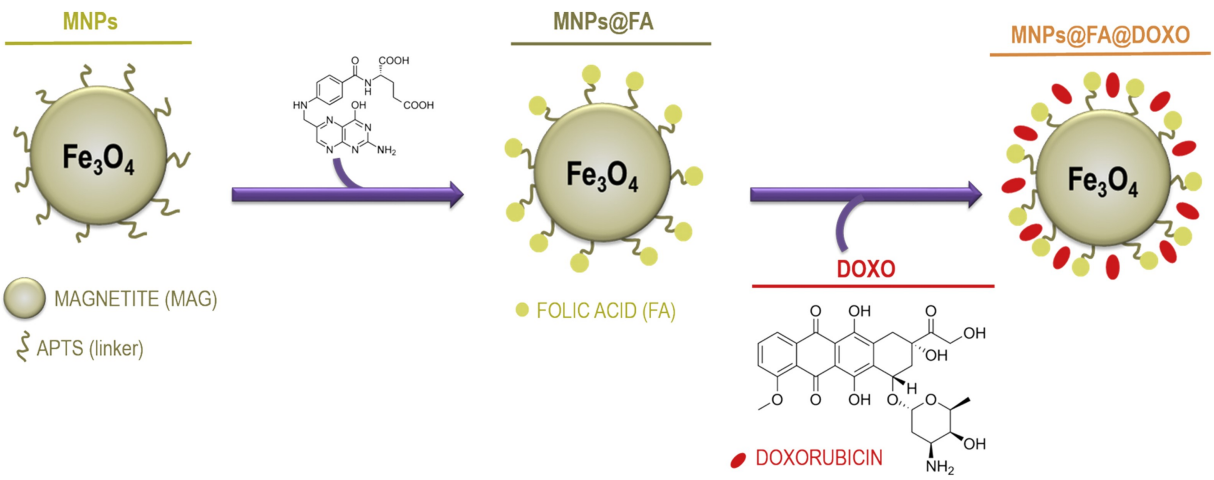
MNPs
MNPs@FA
MNPs@FA@DOXO
Fe_3O_4
Fe_3O_4
Fe_3O_4
DOXO
MAGNETITE (MAG)
APTS (linker)
FOLIC ACID (FA)
DOXORUBICIN

Figure 1

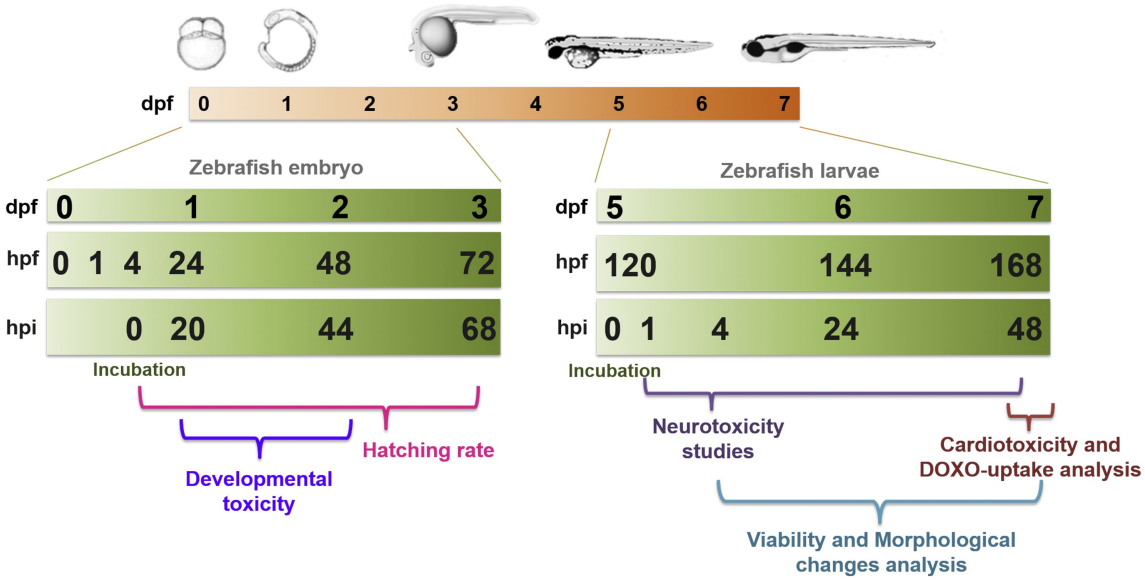
dpf 0 1 2 3 4 5 6 7
Zebrafish embryo
dpf 0 1 2 3
hpf 0 1 4 24 48 72
hpi 0 20 44 68
Incubation
Developmental toxicity
Hatching rate
Zebrafish larvae
dpf 5 6 7
hpf 120 144 168
hpi 0 1 4 24 48
Incubation
Neurotoxicity studies
Cardiotoxicity and DOXO-uptake analysis
Viability and Morphological changes analysis

Figure 2

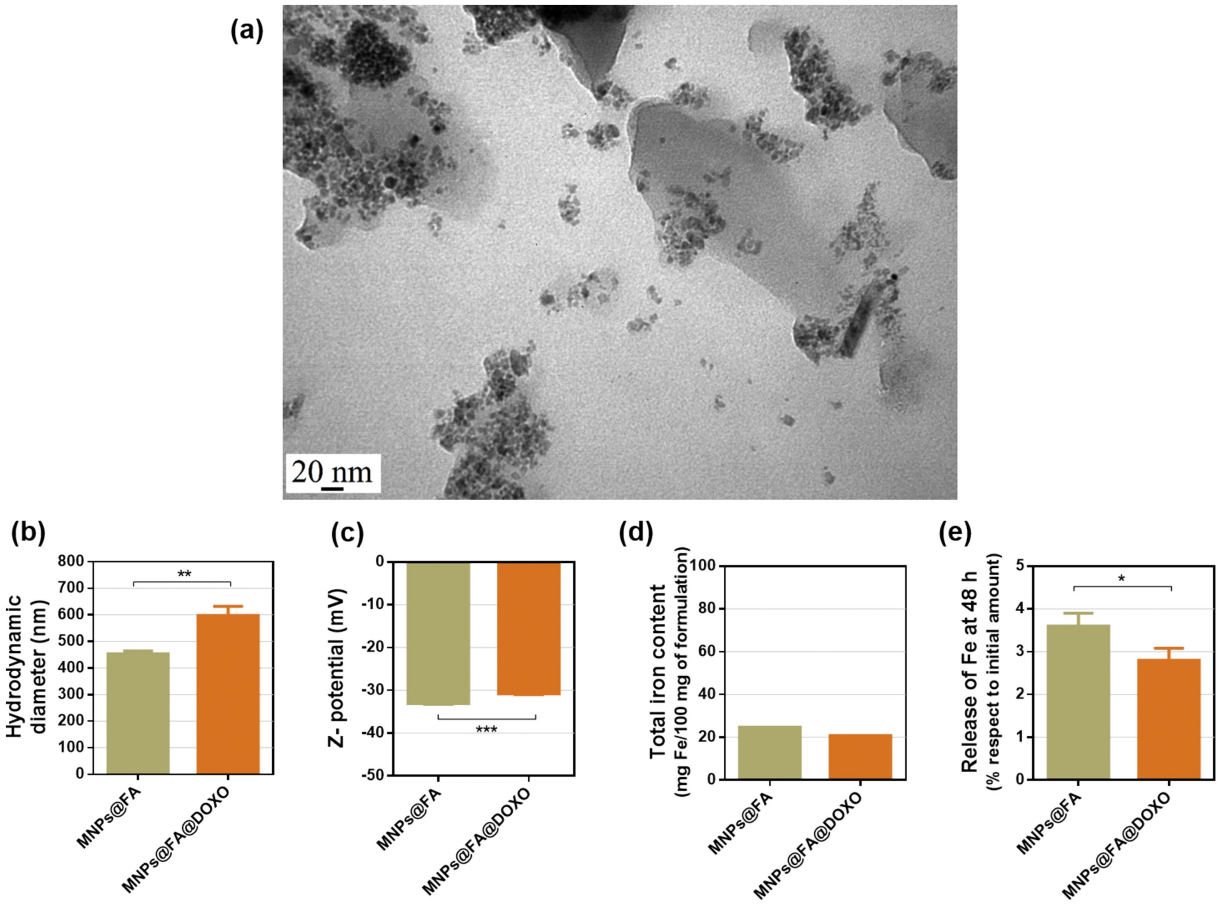
(a)
20 nm
(b)
Hydrodynamic diameter (nm)
800
700
600
500
400
300
200
100
0
**
MNPs@FA
MNPs@FA@DOXO
(c)
Z- potential (mV)
0
-10
-20
-30
-40
-50
***
MNPs@FA
MNPs@FA@DOXO
(d)
Total iron content (mg Fe/100 mg of formulation)
100
80
60
40
20
0
MNPs@FA
MNPs@FA@DOXO
(e)
Release of Fe at 48 h (% respect to initial amount)
5
4
3
2
1
0
*
MNPs@FA
MNPs@FA@DOXO

Figure 3

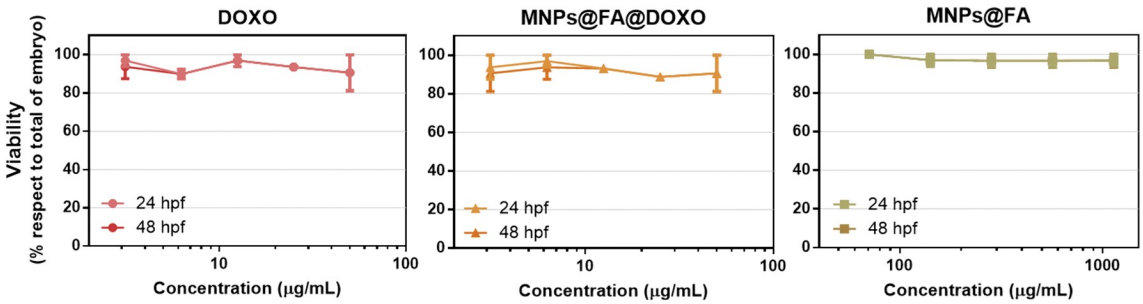
DOXO
MNPs@FA@DOXO
MNPs@FA
Viability
(% respect to total of embryo)
100
80
60
40
20
0
24 hpf
48 hpf
10
100
1000
Concentration (µg/mL)

Figure 4

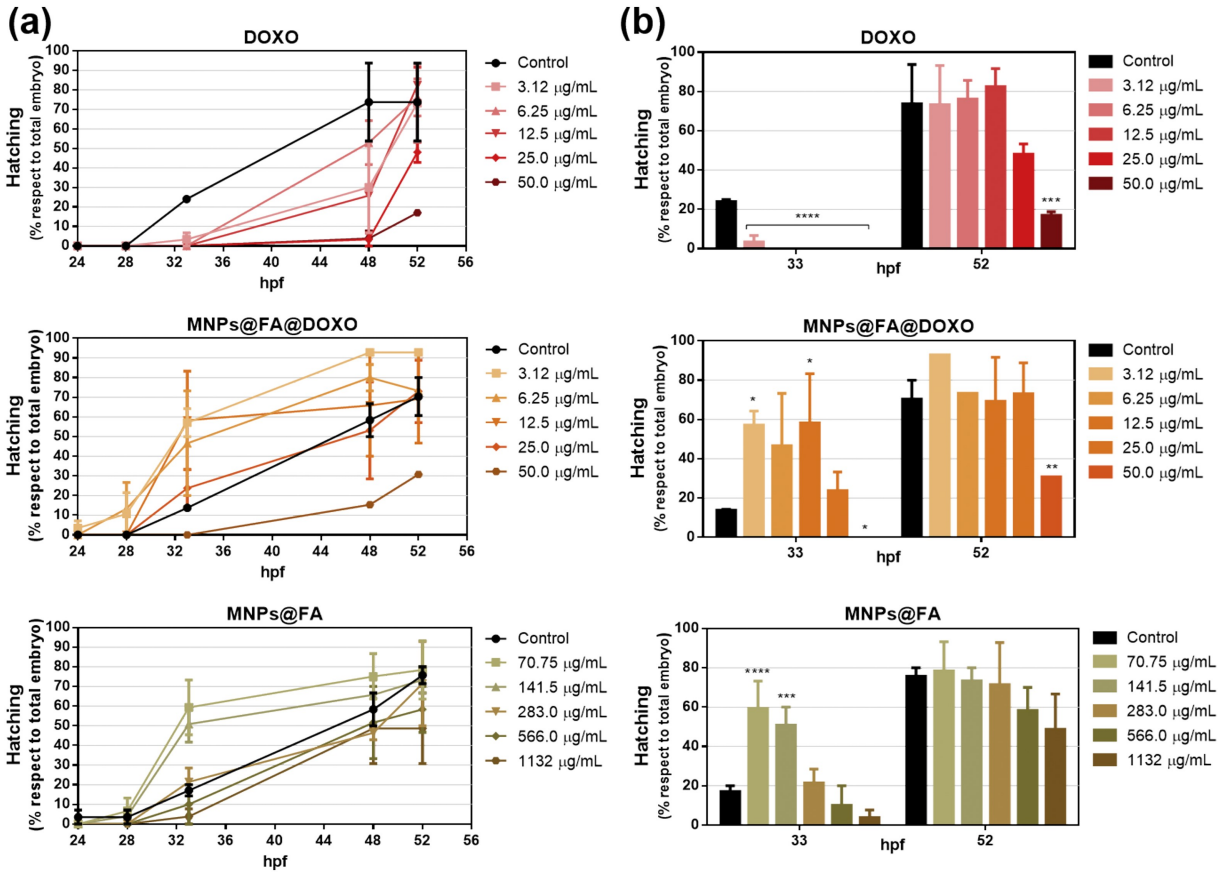
(a)
DOXO
Hatching
(% respect to total embryo)
hpf
Control
3.12 μg/mL
6.25 μg/mL
12.5 μg/mL
25.0 μg/mL
50.0 μg/mL
MNPs@FA@DOXO
MNPs@FA
70.75 μg/mL
141.5 μg/mL
283.0 μg/mL
566.0 μg/mL
1132 μg/mL
(b)
****
***
*
**

Figure 5

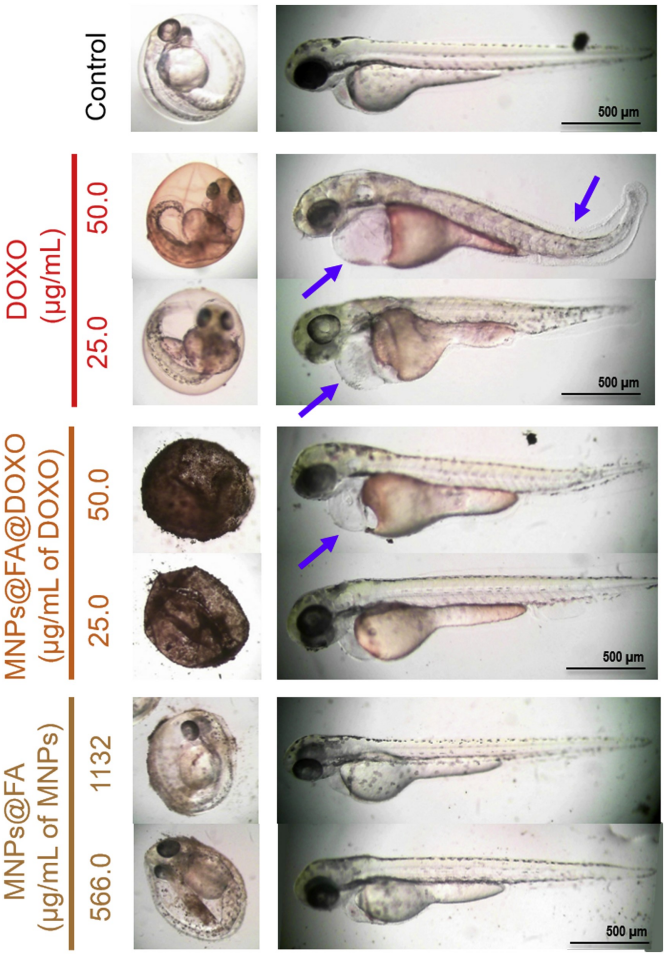
Control
DOXO (µg/mL)
50.0
25.0
MNPs@FA@DOXO (µg/mL of DOXO)
50.0
25.0
MNPs@FA (µg/mL of MNPs)
1132
566.0
500 µm
500 µm
500 µm
500 µm

Figure 6

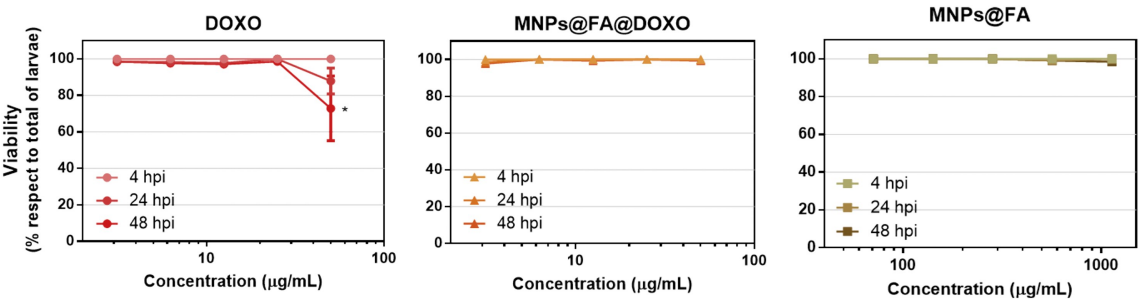
DOXO
MNPs@FA@DOXO
MNPs@FA
Viability
(% respect to total of larvae)
100
80
60
40
20
0
4 hpi
24 hpi
48 hpi
*
10
100
1000
Concentration (μg/mL)

Figure 7

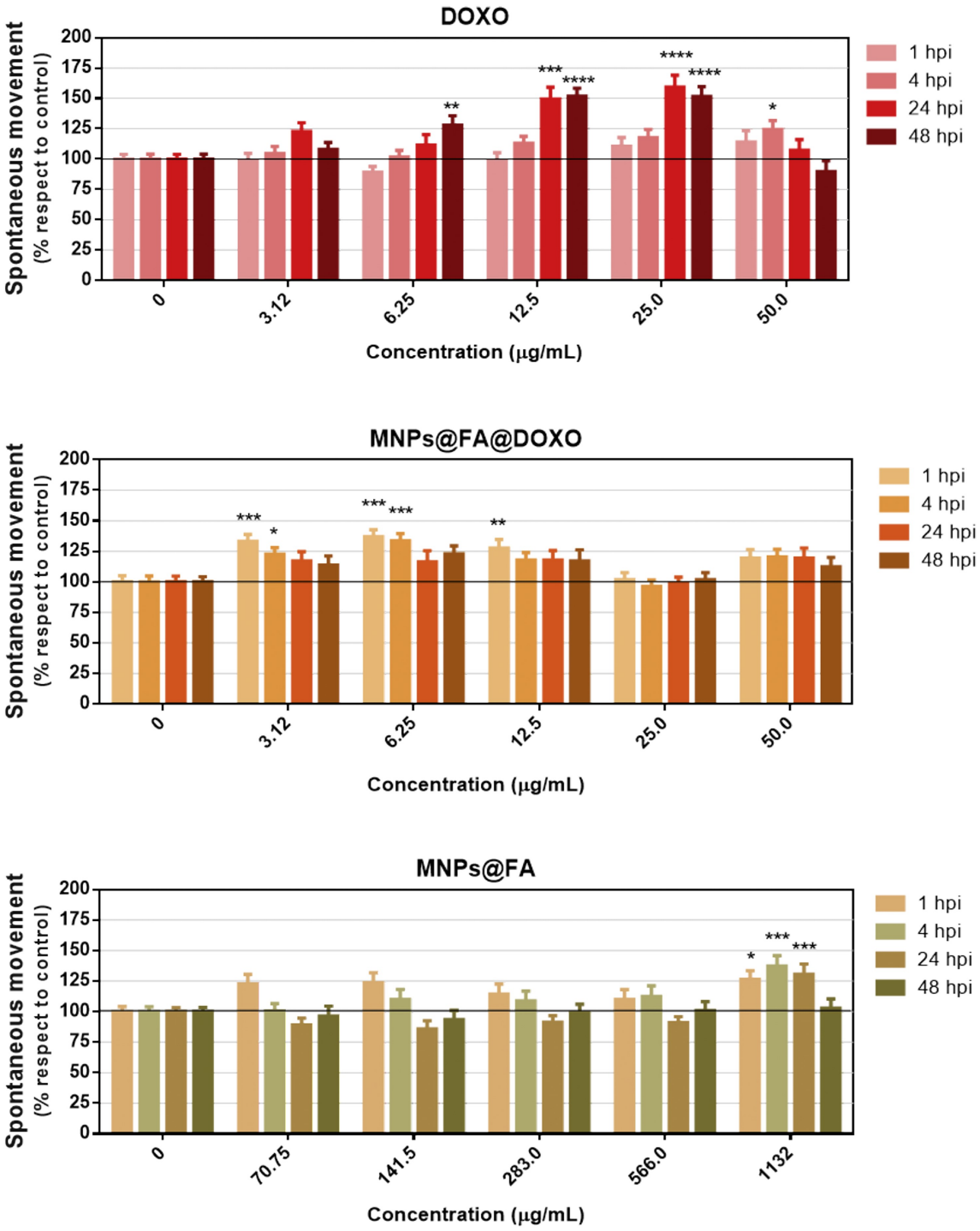
DOXO
Spontaneous movement
(% respect to control)
200
175
150
125
100
75
50
25
0
0
3.12
6.25
12.5
25.0
50.0
Concentration (μg/mL)
1 hpi
4 hpi
24 hpi
48 hpi
**
***
****
****
****
*
MNPs@FA@DOXO
Spontaneous movement
(% respect to control)
Concentration (μg/mL)
1 hpi
4 hpi
24 hpi
48 hpi
***
*
***
***
**
MNPs@FA
Spontaneous movement
(% respect to control)
0
70.75
141.5
283.0
566.0
1132
Concentration (μg/mL)
1 hpi
4 hpi
24 hpi
48 hpi
*
***
***

Figure 8

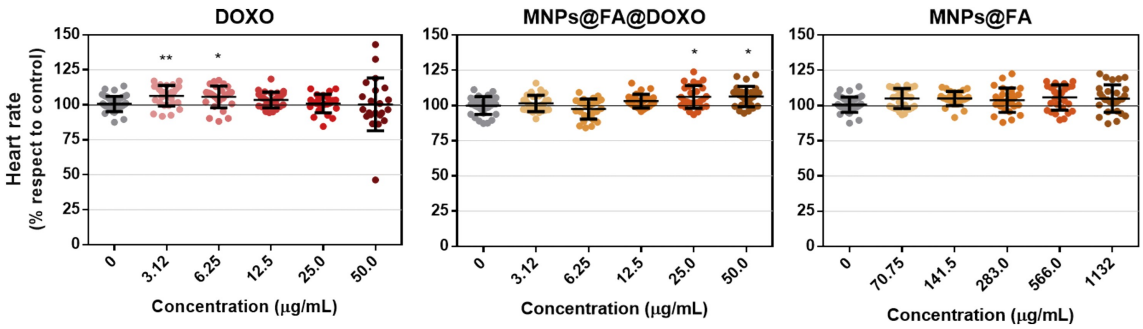
DOXO
MNPs@FA@DOXO
MNPs@FA
Heart rate
(% respect to control)
150
125
100
75
50
25
0
**
*
*
*
0
3.12
6.25
12.5
25.0
50.0
70.75
141.5
283.0
566.0
1132
Concentration (μg/mL)

Figure 9

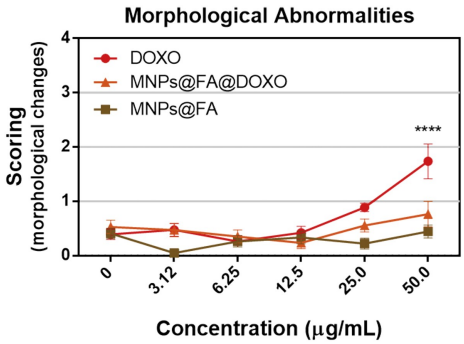
Morphological Abnormalities
DOXO
MNPs@FA@DOXO
MNPs@FA
Scoring
(morphological changes)
****
0
3.12
6.25
12.5
25.0
50.0
Concentration (μg/mL)

Figure 10

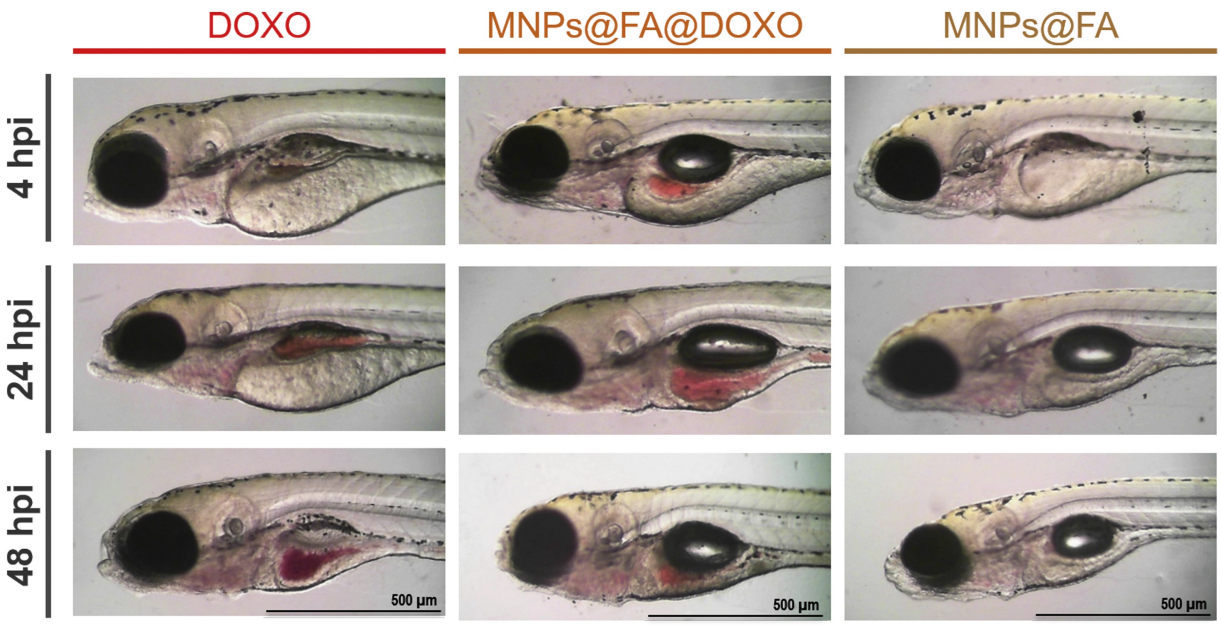

Figure 11

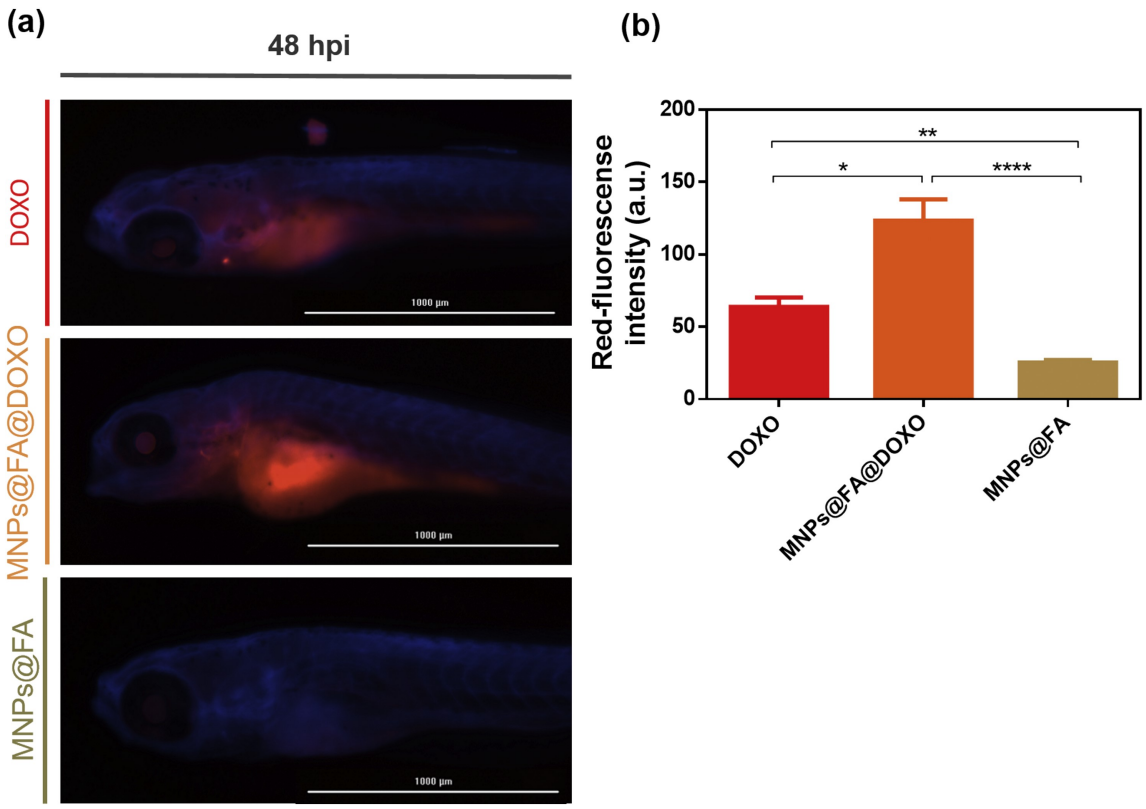
(a)
48 hpi
DOXO
MNPs@FA@DOXO
MNPs@FA
1000 µm
1000 µm
1000 µm
(b)
Red-fluorescense intensity (a.u.)
200
150
100
50
0
**
*
****
DOXO
MNPs@FA@DOXO
MNPs@FA

Figure 12