

Treatment of *Francisella* infections via PLGA- and lipid-based nanoparticle delivery of antibiotics in a zebrafish model

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ABSTRACT: We tested the efficiency of 2 different antibiotics, rifampicin and oxolinic acid, against an established infection caused by fish pathogen *Francisella noatunensis* ssp. *orientalis* (*F.n.o.*) in zebrafish. The drugs were tested in the free form as well as encapsulated into biodegradable nanoparticles, either polylactic-co-glycolic acid (PLGA) nanoparticles or nanostructured lipid carriers. The most promising therapies were PLGA-rifampicin nanoparticles and free oxolinic acid; the PLGA nanoparticles significantly delayed embryo mortality while free oxolinic acid prevented it. Encapsulation of rifampicin in both PLGA and nanostructured lipid carriers enhanced its efficiency against *F.n.o.* infection relative to the free drug. We propose that the zebrafish model is a robust, rapid system for initial testing of different treatments of bacterial diseases important for aquaculture.

KEY WORDS: Nanoparticles · *Francisella orientalis* · Infection · Zebrafish embryo · Antibiotics · *Danio rerio*

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INTRODUCTION

Zebrafish *Danio rerio* are widely used as a vertebrate model for developmental biology but are also increasingly used for studying infectious diseases. Zebrafish are small, have a short generation time and high fertility, are inexpensive compared to rodents, and are relatively easy to maintain. The transparency of zebrafish embryos allows for high-resolution imaging of infections caused by fluorescently labeled pathogens, and of host–pathogen interactions in real time, from the first minutes post infection up to several days (Ko et al. 2011, Gratacap & Wheeler 2014). Moreover, pathogenesis of many bacterial species in zebrafish closely

reflects that of diseased wild and farmed fish and even humans (Phelps & Neely 2005, Sullivan & Kim 2008, Meijer & Spaink 2011, Rowe et al. 2014).

Brudal et al. (2014) established zebrafish embryo infection models of 3 Gram-negative intracellular *Francisella* spp. bacteria at different temperatures. One of the species, *Francisella noatunensis* ssp. *orientalis* (*F.n.o.*) caused the most severe infection in embryos, most likely because it grows best at 28°C, the optimal temperature for zebrafish. *F.n.o.* is an intracellular pathogen of 'warm water' commercially important fish species, such as Nile tilapia, with a global market worth more than 5 billion USD in 2012 (FAO 2012).

F.n.o. expressing a red fluorescent mCherry protein (*F.n.o.*-mCh) allowed live imaging of zebrafish embryos. Within hours after injecting the bacteria, they were actively taken up by the fish macrophages, which then assembled into granuloma-like structures. The infection led to a pro-inflammatory immune response dominated by the up-regulation of the pro-inflammatory cytokines TNF- α and IL-1 β , followed by rapid mortality of the infected embryos within 3 to 5 d (Brudal et al. 2014).

Treatment options for *Francisella* infections in aquaculture are limited, in part due to the lack of effective vaccines and suitable methods of drug administration. The US Food and Drug Administration (FDA) allows only 3 antibiotics for use in aquaculture in the USA: oxytetracycline (Terramycin 200; Phibro Animal Health), ormetoprim-sulfadimethoxine (Romet-30 type A; Pharmaq AS), and florfenicol (Aquaflor type A; Intervet/Schering-Plough). The latter is the only one that has been tested against *F.n.o.*, and its minimal inhibitory concentration was determined to be 2 $\mu\text{g ml}^{-1}$ (Soto et al. 2010). No other reports on the susceptibility of *F.n.o.* to antibiotics have been published to date.

The treatment of infections in aquaculture is often performed using antibiotics in the free form, added to the water or directly into the fish feed. This practice is associated with development of drug-resistant bacteria and other environmentally detrimental side effects. Moreover, drugs in the free form can have limitations such as poor bioavailability and high toxicity. To overcome these limitations, novel strategies have been initiated to develop nano-sized drug-carrier systems. The carriers can protect the drugs from premature degradation, improve their bioavailability and pharmacokinetics (sustained release), and enhance their poor cell penetration, low water solubility, or antimicrobial efficiency, due to targeted delivery (Couvreux & Vauthier 2006, Danhier et al. 2012).

Here we focused on 2 biodegradable drug delivery systems: polymeric nanoparticles (NPs) and lipid NPs (Sawant & Dodiya 2008). The polymer-based NPs are usually made of high molecular weight carriers in which the drug is embedded or covalently bound to the polymer matrix. In contrast, lipid-based systems are self-assembled structures made of 1 or several lipid molecules; these can range from liposomes to more complex nanostructured lipid carriers (NLCs). NLCs combine the advantages of polymeric NPs and successfully overcome some of their drawbacks (Müller et al. 2000, 2002). In this context, NLCs show high drug-loading capacity, high stability, do not

require the use of organic solvents during production, and are inexpensive to produce and easy to scale up (Müller et al. 2000, Müller & Keck 2004). In addition, NLCs are made of endogenous lipids or lipids similar to those existing in the body, and are biocompatible, biodegradable, and non-toxic (Mehnerdt & Mader 2001).

The biocompatible, biodegradable, FDA-approved copolymer of lactic acid and glycolic acid (PLGA) is one of the most widely explored classes of hydrophobic polymers. It is used for fabrication of devices for drug delivery and vaccine candidates for human as well as for aquaculture use, both in commercial and in research applications (Jiang et al. 2005, Lu et al. 2009, Makadia & Siegel 2011).

Studies of antibiotic-loaded PLGA NPs in several animal models have shown their potential for the treatment of infections. NPs are particularly desirable against intracellular bacteria that reside in phagocytic cells such as macrophages; these particles accumulate preferentially inside infected cells, whose main biological function is to phagocytose, and thereby remove, foreign particles from the circulation (Italia et al. 2009, Toti et al. 2011, Danhier et al. 2012). For example, the effectiveness of drug (mostly rifampicin, RIF) loaded PLGA particles against the intracellular pathogen *Mycobacterium tuberculosis* has been demonstrated in mouse, rat, guinea pig, rabbit, and monkey models (Suarez et al. 2001, Griffiths et al. 2010).

Fenaroli et al. (2014) extended this NP approach by using *M. marinum*, a close relative of *M. tuberculosis*, in the zebrafish model. Fluorescent NPs were targeted to the infected macrophages while the NPs enclosing RIF gave an efficient response, as evaluated by zebrafish embryo survival and by quantification of fluorescent bacteria. RIF in NPs was more effective than the drug in free form. In that study, RIF immersion bath was ineffective, although this administration route has shown some efficacy when using other antibiotics in the same model (Adams et al. 2011).

A major goal of the present study was to apply RIF and oxolinic acid (OXA) treatment strategies against *Francisella* infection in the zebrafish model. RIF is a hydrophobic drug used as a standard in treatment of intracellular infections in research-based and medical care institutions, whereas OXA is a hydrophilic substance widely used in aquaculture in many countries but has unwanted side effects, such as altering the system, for example by up-regulating the transcript levels of pro-inflammatory cytokines in several fish species (Romero et al. 2012). We evaluated the

antimicrobial efficiency of RIF and OXA in the free form and in 2 carrier systems, PLGA NPs and NLCs. We expected that these drugs will have different properties both in the free form and when encapsulated into the carriers, and the efficiency of the treatments was of interest. We also took advantage of the fact that the zebrafish embryo model is a highly sensitive indicator of drug toxicity.

MATERIALS AND METHODS

PLGA NP and NLC preparation

PLGA-RIF NPs were prepared by single water-in-oil-in-water emulsion solvent evaporation method and characterized by electron microscopy as described by Kalluru et al. (2013).

NLCs were made using high shear homogenization and ultra-sonication techniques. In detail, Precirol ATO 5 (Gattefossé) (37% w/w for NLC-RIF or 47% w/w for NLC-OXA), miglyol-812 (Acofarma) (26% w/w), polysorbate 60 (Merk) (17% w/w) and RIF (Sigma-Aldrich) (20% w/w) or OXA (Sigma-Aldrich) (10% w/w) were heated in a water bath to 70°C. When the solid lipid was melted, ultrapure water ($T=70^{\circ}\text{C}$) was added (with liquid lipid and surfactant) (NLC), with Coumarin 6 (NLC-probe), RIF (NLC-RIF), or OXA (NLC-OXA). The mixture was passed through an Ultra-Turrax T25 (Janke and Kunkel IKA-Labortechnik) at 3500 rpm for 30 s, followed by sonication using a Sonics and Materials Vibra-Cell™ CV18 at 70% power for 5 min, resulting in a nanoemulsion that was cooled to room temperature and stored.

Efficiency of drug encapsulation

The efficiency of encapsulation of RIF and OXA into NLC was determined by UV/Vis spectrophotometry (Jasco) at 334 and 262 nm, respectively. The samples were diluted in Milli-Q water (1:200). The resulting solutions were dissolved in 400 μl of acetonitrile (ACN) and vigorously vortexed for 30 min to completely extract drugs from the NLCs. The solutions were then transferred into Ultrafree Centrifugal Filter Devices (Millipore). Centrifugation was performed using a Jouan BR4i multifunction centrifuge with a KeyWrite-D interface (Thermo Electron) with a fixed 23° angle rotor and 3000 $\times g$ spin (8 min at 20°C). The RIF and OXA released from the particles were present in the supernatant, which was stored in

the centrifuge tube and quantified. The encapsulation efficiency (EE) was defined by the ratio of measured and initial amount of the drugs encapsulated (Danhier et al. 2009) and was calculated as:

$$\text{EE} = \frac{\text{weight of drug encapsulated}}{\text{weight of drug initially}} \times 100\% \quad (1)$$

Loading of PLGA NPs was quantified using HPLC-UV. For both particle types, 1 mg ml⁻¹ solutions were made in ACN (HiPerSolv Chromanorm, VWR) and briefly ultrasonicated. The samples were centrifuged (13 000 $\times g$, 1 min), and 10 μl of the supernatant was injected into the system by an Agilent 1200 series GL1377A micro WPS auto sampler. When necessary, the samples were diluted 10 times before the injection. RIF was separated with an ACE3 C18 column (150 mm \times 4.6 mm inner diameter; Advanced Chromatography Technologies). An Agilent 1100 series GL1378 pump (Agilent Technologies) was used to deliver a mobile phase at a flow rate of 1 $\mu\text{l min}^{-1}$. The mobile phase consisted of 70% ACN and 0.1% formic acid in water. The analytes were detected using an Agilent 1200 series GL1365D MWD detector set to 480 nm.

Zeta potential and size measurements

The zeta potential and size measurements of PLGA NPs were performed on a Malvern Zetasizer Nano ZS (Malvern Instruments). The parameters were determined by the electrophoretic mobility of the samples at 25°C, using laser Doppler velocimetry. Each experiment was repeated 3 times, and the average values are presented. PLGA-RIF NPs were re-suspended at 1 mg ml⁻¹ in distilled water prior to measurement. The zeta potential of NLCs was determined by measurement of the electrophoretic mobility using a BI-MAS dynamic light scattering (DLS) instrument (Brookhaven Instruments). Prior to measurements, samples were diluted (1:100) in Milli-Q water. For each measurement, 10 runs (each with 10 cycles) were performed at 20°C. NLC size was measured using DLS, also known as photon correlation spectroscopy, using a BI-MAS DLS instrument (Brookhaven Instruments), operating at a scattering angle of 90°. Prior to the measurements, samples were diluted (1:100) in Milli-Q water and filtered with a syringe filter (800 nm). DLS data were analyzed at 20°C with a dust cut-off set to 30. The mean hydrodynamic diameter (Z -average) and the polydispersity index (PI) were determined as a measure of

the width of the particle size distributions. At each measurement, 6 runs of 2 min each were performed.

Preparation of bacteria for microinjections

The *Francisella noatunensis* ssp. *orientalis* strain routinely used in these experiments expresses a fluorophore (mCherry) and kanamycin resistance gene (Brudal et al. 2014). Frozen stock was inoculated on Eugon chocolate agar plates (Brudal et al. 2013), and once the colonies were formed, they were transferred into liquid Eugon broth supplemented with iron (Sigma-Aldrich). Overnight cultures were collected by centrifugation at $12\,800 \times g$ (5 min at room temperature). The pellet was re-suspended in phosphate-buffered saline (PBS), pH 7.4. Optical density at 600 nm was measured with Genesys 20 Spectrophotometer (ThermoFisher Scientific) and adjusted to the value of 1.0. This corresponds to approximately 4.5×10^3 colony-forming units being injected into each embryo, as estimated by Brudal et al. (2014). The control zebrafish embryo group was injected with PBS. An aliquot of phenol red sodium salt (Sigma-Aldrich) stock solution was added to the prepared bacterial suspensions in PBS to a final concentration of 0.01% v/v to ensure visualization of the injection.

Preparation of therapeutic agents for microinjections

The lyophilized PLGA-RIF NPs were re-suspended in PBS, pH 7.4 by bath sonication for 1 to 2 min. The concentration of the PLGA-RIF NP suspension used for injections was 16 mg ml^{-1} (this corresponds to 5.3 mg ml^{-1} of RIF). The estimated amount of RIF per embryo was approximately 14.4 ng. This concentration was chosen as a result of previous successful therapy against tuberculosis in a mouse model (Pandey & Khuller 2004). The concentration of RIF and OXA in NLC preparations was 14.6 and 7.4 mg ml^{-1} , respectively. The injection volume was adjusted to deliver similar amounts of antibiotics into zebrafish embryos in the case of PLGA nanoparticles and NLC.

Antibiotic baths

The concentrations of antibiotics in baths were chosen based on previous experiments. We dissolved $100 \text{ } \mu\text{g ml}^{-1}$ of OXA in embryo water and $100 \text{ } \mu\text{g ml}^{-1}$

of RIF–1% v/v dimethyl sulfoxide (DMSO) in zebrafish embryo water (Westerfield 2000), as RIF is only partially soluble in water. The presence of DMSO in the embryo water did not affect zebrafish health and development (data not shown). These concentrations were chosen based on preliminary results of bath treatment experiments. The solutions were stored at 4°C , and the baths were refreshed every day. RIF solution was additionally protected from light, as the drug is light sensitive.

Zebrafish embryo maintenance

Zebrafish wild-type strain AB and transgenic strain Tg(mpeg1:mCherry) embryos were bred in the in-house facility with a 12:12 h light:dark cycle. All zebrafish adults and embryos were handled according to standard protocols, and water quality was monitored daily (Westerfield 2000). Zebrafish embryos were manually dechorionated not earlier than 30 h post fertilization, transferred into fresh embryo water, and kept at 28°C prior to injections.

Zebrafish embryo microinjections

Healthy, normally developed zebrafish embryos were divided into groups of 21 by fish facility members and passed to the experimenter. The embryos in therapeutic groups were injected with the bacterial suspension into blood circulation at 48 h post fertilization followed by the administration of drugs at 6 h post infection (hpi) using a Narishige EG-400 microinjector with an Eppendorf Femtojet controlling unit. The control groups were injected with corresponding volumes of PBS. A detailed description of the procedure was reported earlier (Brudal et al. 2014).

Disease progression and mortality

The number of animals per experimental condition was calculated as 21, which allows achieving the statistical power of 0.95. The survival data were processed using the Kaplan-Meier method with GraphPad Prism 6.0 for Windows (GraphPad Software). The number of embryos used for disease score studies was reduced to 12, the minimum number of animals at which a statistical difference between the groups could be achieved. The morphological observations during the infection were performed in a blind fash-

ion. Twelve embryos per group were put into separate wells of 12-well plates, and 8 parameters were evaluated by a person not involved in the current study. Physiological parameters, including response to touch, swimming behavior, and equilibrium, were noted first. Further, MS-222 (tricaine methanesulfonate; pH 7.4) solution was added to a final concentration of 80 $\mu\text{g ml}^{-1}$ followed by evaluation of morphological parameters (embryo body shape, blood flow, heartbeat, edema presence/absence, and swim bladder size). After examination, the embryos were transferred to fresh embryo water. Each of the 8 parameters was given a value from 0 (normal) to 2 (abnormal), and then all values per embryo were summed; thus, every embryo was scored with a collective number from 0 to 16 adapted from Palha et al. (2013). The disease score for morbid or terminally ill fish had high score numbers, and low score numbers close to 0 characterized healthy individuals.

Statistical analysis

Kaplan-Meier representation and a log-rank (Mantel-Cox) test were used to assess differences between the groups in survival experiments. In the disease score evaluation, a 2-tailed unpaired test, or an analysis of variance (ANOVA) followed by Bonferroni multiple comparison test, was used to evaluate difference between means (with a 95% confidence interval). Statistical analysis and plotting the graphs were performed using GraphPad Prism 6 software (GraphPad Software).

Imaging of zebrafish embryos

Fluorescent imaging was performed with a Leica AF 6000 (Leica Microsystems). The embryos were anesthetized and embedded into low-melting-point agarose prior to imaging. The micrographs were taken using Leica Application Suite V4.0.0 and processed on the Fiji platform.

Ethical regulations

All experiments on zebrafish were carried out in agreement with the Animal Protection Act and the Regulation on Animal Experimentation (lovdata.no), which complies with EU

regulations. Experimental protocols were approved by the Norwegian Animal Research Authority (Forsøksdyrutvalgets tilsyns- og søknadssystem, FOTS), approval ID is 6050.

RESULTS

The main characteristics of the NPs we used are summarized in Table 1. The size of NPs and NLCs was in the range of 200–500 nm. The suspension of the latter was monodisperse with a PI of about 0.1 (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d125p019_supp.pdf). For PLGA NPs, this parameter was in the range of 0.2–0.4, which are common values for the particles prepared by the emulsion solvent evaporation method (Gaumet et al. 2008, Danhier et al. 2009).

The average size and polydispersity of the formulations made them suitable for the administration via intravenous injections into zebrafish embryos (Moore et al. 2006). NLCs have a higher negative zeta-potential than PLGA NPs, and therefore they can be considered physically more stable due to the electrostatic repulsions between particles.

All synthesized NPs possessed a negative surface charge, an advantage since these are generally less toxic than NPs with a positive charge (Kedmi et al. 2010). NLCs had about 2-fold higher encapsulation efficiency of RIF than PLGA NPs. Moreover, when using our routine method, it was possible to encapsulate OXA efficiently into NLCs, but not into PLGA.

Treatment of *F.n.o.* infection with RIF and OXA

Brudal et al. (2014) showed that *F.n.o.* labeled with mCherry (*F.n.o.*-mCh) were mainly phagocytosed by macrophages. When nanocarriers, i.e. PLGA NPs and NLCs with encapsulated Coumarin 6, were

Table 1. Properties of PLGA nanoparticles (NPs) and nanostructured lipid carriers (NLC). All parameters are represented by their average (\pm SD) value obtained from at least 3 multiple measurements. ZP: zeta potential; RIF: rifampicin; OXA: oxolinic acid; PI: polydispersity index; EE: encapsulation efficiency (see Eq. 1)

NP type	ZP (mV)	Diameter (nm)	PI	EE (%)	Drug con. (mg ml^{-1})
PLGA	-14.8 ± 3.9	527 ± 97	0.4	–	–
PLGA-RIF	-20.7 ± 0.7	464 ± 129	0.2	31.8	5.3
NLC-RIF	-26.1 ± 0.7	251 ± 12	0.1	92	14.6
NLC-OXA	-32.0 ± 0.5	193 ± 8	0.1	93	7.4
NLC	-35.9 ± 0.6	266 ± 14	0.1	–	–

injected approximately 2 h after the bacteria, the majority of them were found to be co-localized with the bacteria shortly after the injection (Fig. 1). This demonstrates a great strength of the NP treatment, namely passive targeting.

The antimicrobial activity of RIF and OXA was tested prior to the current study, both *in vitro* at a minimum amount of 2 µg and in a zebrafish embryo–francisellosis model (data not shown). The *in vivo* study showed that groups of embryos immersed in OXA and RIF baths with concentrations of 100 µg ml⁻¹ at 24 hpi reached 20 and 60% mortality, respectively. Although OXA was efficient in the immersion bath treatment, it has several side effects, as mentioned above.

We therefore formulated a delivery system for OXA. NLC is more suitable than PLGA, as OXA is hydrophilic and unsuitable for the hydrophobic

PLGA. We therefore compared the antimicrobial effect of OXA in free form to the encapsulated form. In contrast, PLGA NPs are well-suited for hydrophobic RIF, extensively used in the treatment of infections caused by *Mycobacteria* (Suarez et al. 2001).

The preliminary PLGA-RIF NP treatment experiment where the NPs were injected at 24 hpi demonstrated that this therapy could delay the mortality of zebrafish embryos and resulted in 20% survival by the end of the experiment (6 dpi). A brief evaluation by microscopy showed a decrease of *F.n.o.-mCh* burden in the embryos treated with PLGA-RIF NPs compared to the untreated ones. Fig. 2 shows the typical pattern seen in PLGA-RIF-treated groups, revealing a considerable decrease in the total fluorescent signal from the bacteria over time, although in no experiment was a complete clearance of infection achieved in all of the infected embryos (Fig. 2).

One of the possible reasons for the lack of complete treatment could be that RIF is released from PLGA NPs too slowly. Therefore, we reduced the duration of the therapies against *F.n.o.-mCh*, to 6 instead of 24 h. As estimated by qPCR analysis, the bacterial burden in this case was about 2.5-fold lower than previously and corresponded to approximately 9×10^3 cells embryo⁻¹ (Fig. 3) (Brudal et al. 2014).

For the present experiment, either bath immersion or NP injection was administered at 6 hpi. In the bath experiments, the solutions were changed daily until complete mortality of all groups was observed. In contrast, only 1 injection of the NPs was performed including groups of uninfected embryos and of infected embryos left without treatment (injected with PBS). The injection of empty NPs did not affect embryo survival (data not shown). Additionally, we included NLCs with encapsulated RIF as a treatment strategy in this experiment. We concluded that that lipid-based NLCs would release their content faster than polymeric PLGA-RIF NPs (Fig. S2 in the Supplement), which might be sufficient to clear the bacterial burden developed over 6 h (Reithmeier et al. 2001, Mohammad & Reineke 2013). In the various treatment options tested, we therefore

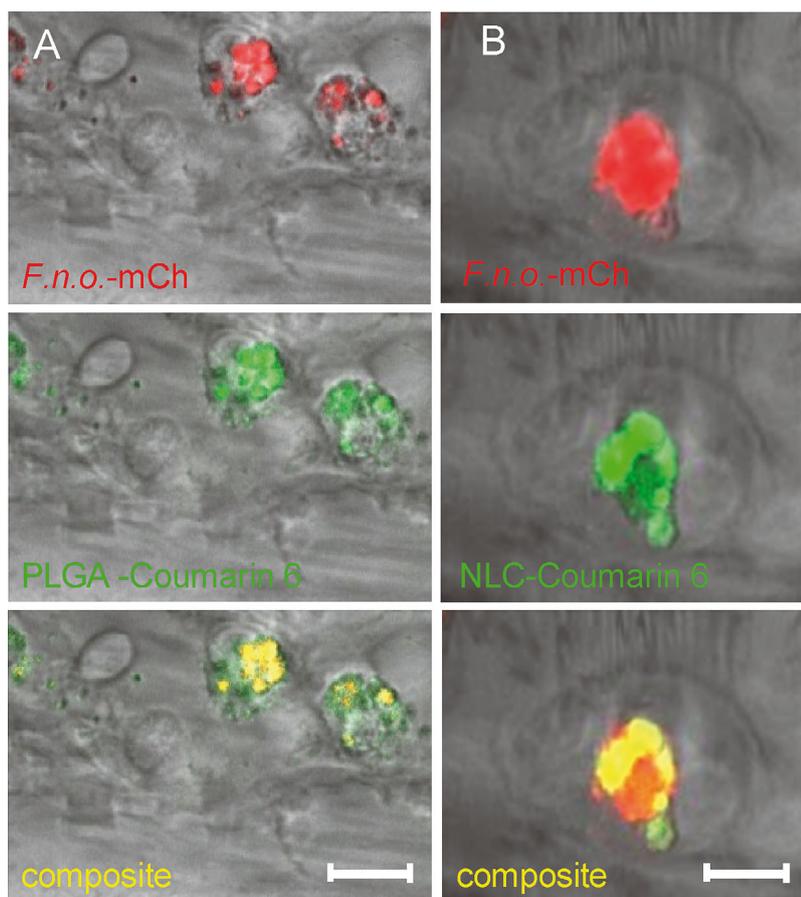


Fig. 1. Co-localization of nanoparticles (NPs) and bacteria. Zebrafish *Danio rerio* embryos were injected with red fluorescent bacteria *Francisella noatunensis* ssp. *orientalis* labeled with mCherry (*F.n.o.-mCh*), followed by the injection of either (A) PLGA or (B) nanostructured lipid carrier (NLC) NPs labeled with Coumarin 6. The imaging of live zebrafish was performed 1–2 h after injection of NPs, or 3–4 h after injection of the bacteria. The bulk of bacteria and NPs were co-localized in macrophages. Scale bar = 10 µm

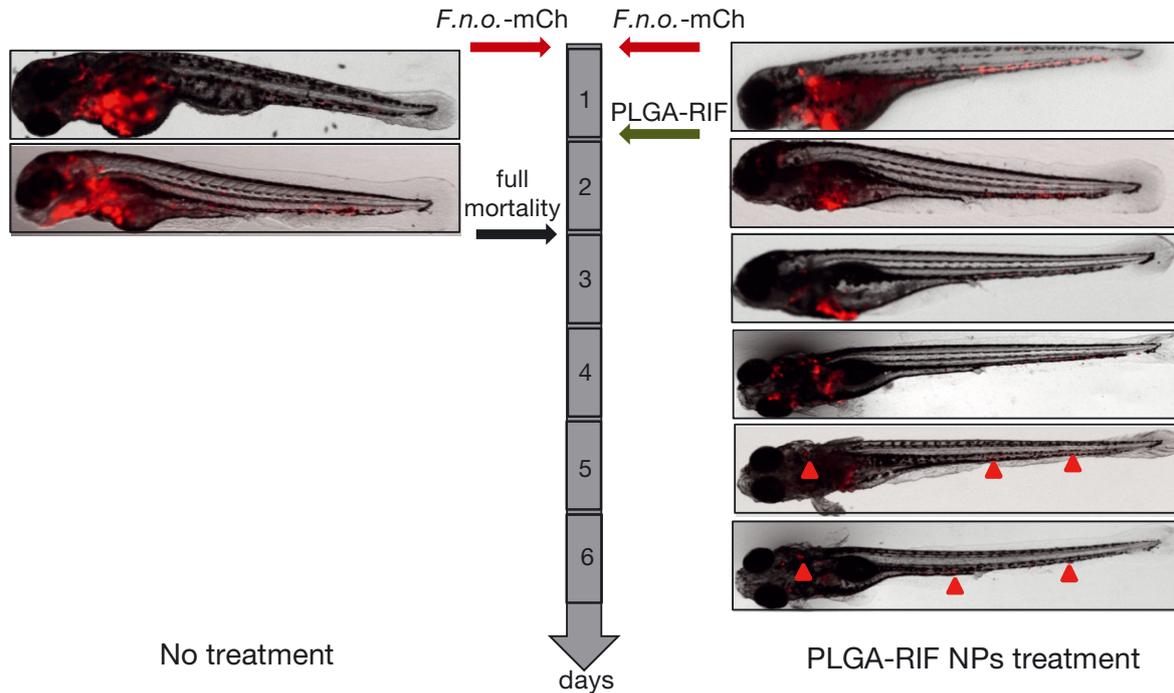


Fig. 2. Schematic representation of the experiment *Francisella noatunensis* ssp. *orientalis* labeled with mCherry (*F.n.o.-mCh*) infection, treated by PLGA-rifampicin (RIF) nanoparticles (NPs). Zebrafish *Danio rerio* embryos were injected with the bacteria, and the group which received no treatment reached full mortality in 2 d. The group which was injected with PLGA-RIF NPs 1 d after infection with *F.n.o.-mCh* demonstrated decreased signal of the fluorescent bacteria over time. Complete elimination of the bacteria was not observed in all cases, and several bacterial foci could still be in the embryos 5–6 d post infection (marked with red arrowheads)

assessed their effect in reducing the rate and extent of embryo mortality. In this experiment, we compared the antimicrobial effect of continuous antibiotic bath immersion versus a single injection of PLGA

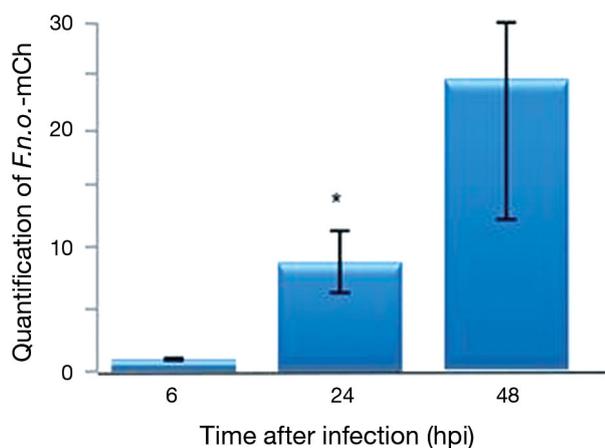


Fig. 3. Reproduction (mean ± SD) of *Francisella noatunensis* ssp. *orientalis* labeled with mCherry (*F.n.o.-mCh*) infecting zebrafish *Danio rerio* embryos at 6, 24, and 48 h post infection (hpi). The asterisk indicates a statistically significant difference between 6 hpi and 24 hpi ($p < 0.05$); error-bars show SD. Adapted from Brudal et al. (2014; their Fig. 1B).
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NPs or NLCs (Fig. 4). The OXA bath demonstrated full protection against *Francisella* infection, whereas the RIF bath of the same concentration could only delay the onset of mortality for 2 d compared to the untreated group. Among NP therapies, the PLGA-RIF treatment was the most efficient, with 40% fish survival at the end of the experiment. NLC-OXA and NLC-RIF treatments were less efficient, and their effects were not statistically different from each other (Table 2). Both drug-free PLGA NPs and NLCs exerted no antimicrobial protection (data not shown). In summary, all therapies demonstrated a certain degree of efficiency against the bacterial infection, which was statistically significant compared to the untreated control embryos.

Comparing the different forms of RIF, we conclude that PLGA-RIF NPs are the most efficient, whereas the effects of free RIF and NLC-RIF did not differ significantly. There was no significant difference between NLC formulations with encapsulated RIF and OXA, both exerting some protection only for up to 8 dpi. The bath with free OXA provided full protection against the infection during the entire experiment, whereas free RIF protected only 10% of fish by 6 dpi.

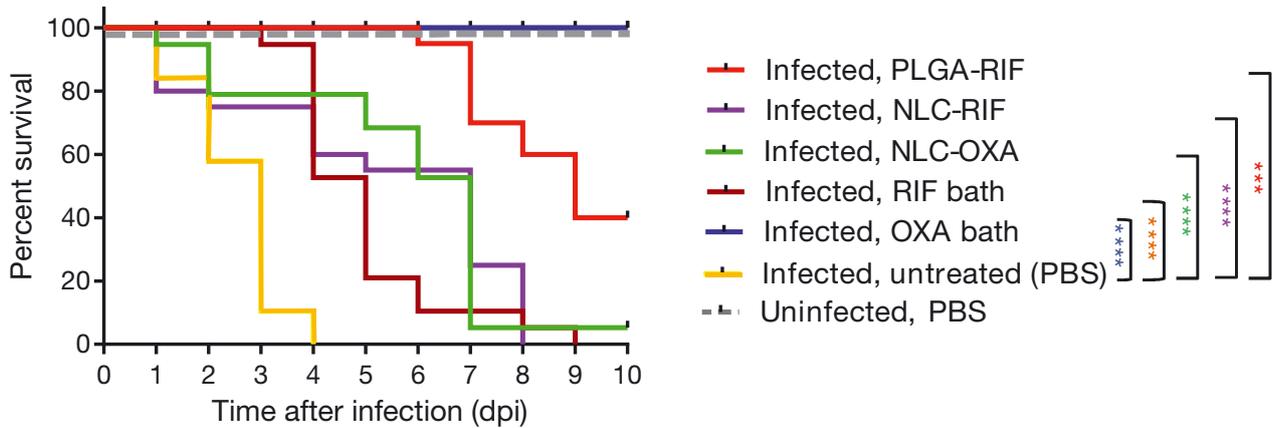


Fig. 4. Kaplan-Meier representation of cumulative survival of zebrafish *Danio rerio* embryos infected with *Francisella noatunensis* ssp. *orientalis* labeled with mCherry (*F.n.o.-mCh*) and treated with different therapies 6 h post infection. Asterisks indicate statistical significance of survival curve differences between each treatment group and the untreated infected control (yellow line): **** $p < 0.0001$; *** $p < 0.001$. Standard errors were removed for simplicity. NLC: nanostructured lipid carrier; RIF: rifampicin; OXA: oxolinic acid; PBS: phosphate-buffered saline

Table 2. Statistical comparison of the therapeutic efficiency between different treatments. Given that survival rates correlate with the therapeutic efficiency, the comparison of survival curves was performed by log-rank (Mantel-Cox) test; **** $p < 0.0001$, * $p < 0.1$, ns: not significant. ↓ / ↑: a treatment listed in the row is less/more efficient than a treatment listed in the column; RIF: rifampicin; OXA: oxolinic acid; NLC: nanostructured lipid carrier

Treatment	PLGA-RIF	NLC-RIF	NLC-OXA	RIF	OXA
PLGA-RIF	–	↓****	↓****	↓****	↑****
NLC-RIF	↑****	–	ns ($p = 0.77$)	ns ($p = 0.27$)	↑****
NLC-OXA	↑****	ns ($p = 0.77$)	–	↓*	↑****
RIF	↑****	ns ($p = 0.27$)	↑*	–	↑****
OXA	↓****	↓****	↓****	↓****	–

Overall disease evaluation

We next addressed the potential toxicity of the various drug treatments by evaluation of physiological and morphological parameters of the larvae previously subjected to treatments. The procedure we followed is described in the Methods section and is shown in Fig. 5A.

The examination was performed at 2 dpi, shortly before the rapid rise in mortality of the untreated group occurred; means are shown in Fig. 5B. A 2-way ANOVA was applied to compare the means, with a confidence interval of 95%. According to this analysis, the mean disease scores of the groups treated with PLGA-RIF NPs and OXA bath were significantly lower than that of the untreated, infected group (Fig. 5B, and see Table S1 in the Supplement). The disease scores for the above antibiotic treatments were both 0, which corresponds to the pheno-

type of healthy embryos. NLC-OXA, NLC-RIF, and RIF bath treated groups of embryos showed high variation in disease scores among individuals.

DISCUSSION

The progression of *Francisella noatunensis* ssp. *orientalis*-mCh infection in zebrafish embryos is a fast process, resulting in full mortality within 3 to 5 dpi; the infected embryos reach 100% mortality by 5–6 d post-fertilization (dpf). This makes the *F.n.o.-mCh*

zebrafish infection model especially attractive for testing therapeutics; moreover, no ethical clearance is needed when embryos are below 6 dpf.

Importantly, we found that drug carriers loaded with fluorescent probes co-localize with the bacteria (Fig. 1). Thus, *F.n.o.* and the NPs were found in the same macrophages via passive targeting. Further, we tested the efficiency of the infection treatment by NPs loaded with OXA or RIF, which exerted antimicrobial activity against *F.n.o.* *in vitro* and in the preliminary bath immersion experiments.

The main parameter we used to evaluate the efficiency of the therapies was the survival rate of the infected zebrafish embryos; this was complemented by fluorescent microscopy analysis of the red fluorescent bacteria and by evaluation of the overall health (disease score) of the fish, using a multi-parameter analysis (Palha et al. 2013). The antibacterial efficiency of the free form of these antibiotics, changed

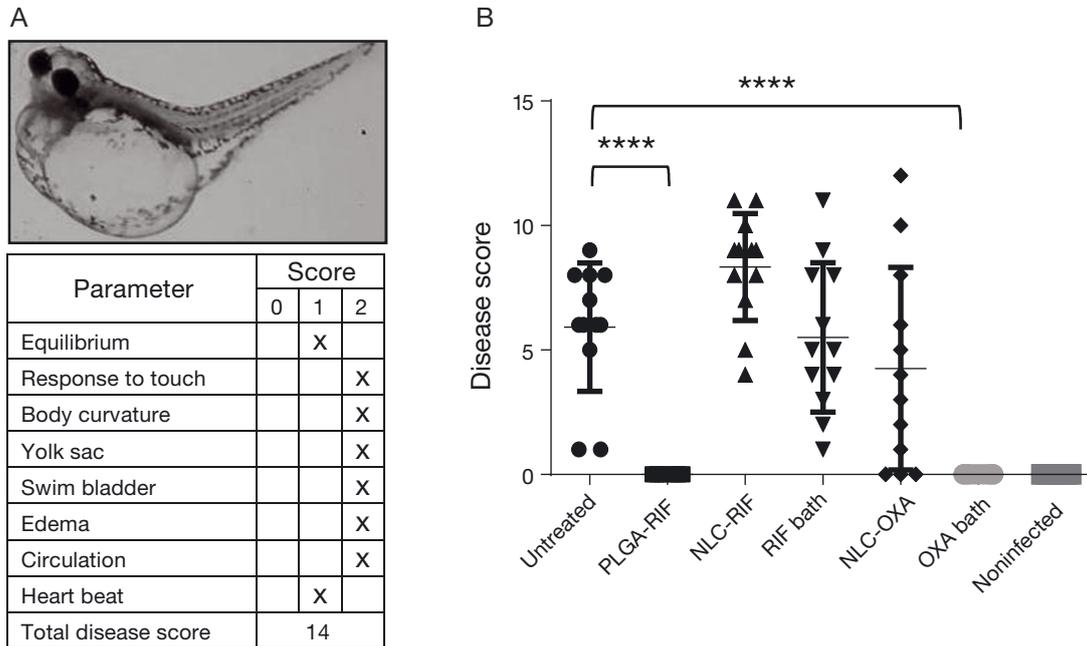


Fig. 5. (A) Disease score test. In this example, the infected zebrafish *Danio rerio* embryo has a disease score of 14, which was calculated using 8 physiological and morphological parameters listed in the table. These parameters were rated from 0 to 2, with a higher score indicating worse condition. All values were summed and resulted in a total disease score of 14. (B) Disease score of the infected zebrafish embryos treated with different therapies, 2 d post infection. The groups treated with PLGA-rifampicin (RIF) and oxolinic acid (OXA) bath were significantly different (**** $p < 0.0001$) from the untreated group, whereas the rest of the therapies were not. The bars indicate mean \pm SEM. NLC: nanostructured lipid carrier

daily, was compared to a single injection of the drugs encapsulated into NPs. Based on all of these criteria, the best therapy overall against *F.n.o.* infection experiment was the bath immersion treatment with OXA, which led to full protection and resulted in healthy embryo phenotypes (low disease score; Table S1, Fig. 5B). A complete bacterial clearance was also confirmed by preliminary fluorescence microscopy analysis. The treatment with free RIF was not as effective as the free OXA, as it was associated with high variation in the disease score among individuals. Both antibiotics exert their effect on bacterial enzymes: RIF inhibits DNA-dependent RNA polymerase, whereas OXA inhibits DNA gyrase (Frontali et al. 1964, Franco & Drlica 1988). Within the framework of our experimental design, we hypothesize that OXA was efficient in the bath because it is more hydrophilic than RIF; moreover, solubilization of the hydrophobic RIF in embryo water necessitated adding 0.1% DMSO, which by itself was well tolerated by embryos.

Encapsulation of OXA into NLC provided more selective uptake of the drug by infected cells. From the outset, we expected that this should decrease the amount of the drug needed to achieve a therapeutic effect and ultimately lead to fewer side effects. The

lipid NP approach seems to be particularly beneficial in the case of OXA, as this drug has a potential to cause bacterial cross-resistance to other antibiotics of the quinolone group, and for this reason it is either used only in moderate amounts (Lunestad & Samuelsen 2008), or even prohibited in many aquaculture settings (Gorbach 2001, Moellering 2005, Sorum 2006). OXA was too hydrophilic to be efficiently encapsulated into PLGA NPs by our routine emulsion solvent evaporation method and therefore only NLC-OXA was used here. However, NLC-OXA was not very effective against *F.n.o.* infection (Fig. 4). This may be attributed to lower stability or a faster degradation time of this type of NP. It has been suggested that lipid particles could be an alternative to polymeric particles when faster content release is desired (Reithmeier et al. 2001). Solid lipid NPs (SLNs) release the most of their content/degrade *in vitro* within 12 to 50 h, depending on their composition and release study conditions (Weyhers et al. 2006, Thatipamula et al. 2011, Baviskar et al. 2012). Since NLCs have a lower degree of crystallinity than the tested SLNs, they are expected to degrade faster than SLNs (Olbrich et al. 2002). Although the NLC-OXA was not as effective as free OXA, we suggest that it is worthwhile to consider other NLCs or poly-

meric NPs for encapsulating this drug for aquaculture since using the free form is undesirable.

A single injection of PLGA-RIF NPs was the second best treatment after free OXA, with regards to embryo survival. The treated embryo group had a low disease score (i.e. was healthy), which was similar to the uninfected embryo group and almost all of the embryos treated with OXA bath. Nevertheless, microscopic analysis showed that PLGA-RIF NP treatment did not result in complete bacterial clearance (Fig. 2), irrespective of whether the bacterial burden was lower (at 6 hpi) or higher (at 24 hpi), although the treatment delayed the onset of mortality in the first case.

Due to the inability of PLGA-RIF NPs to clear the infection in all of the embryos, regardless of the treatment time (6 or 24 hpi), we encapsulated RIF into lipid NPs and tested them in the zebrafish model. The NLC-RIF treatment was significant, but not highly effective, and not distinguishable from the effects of free RIF (Fig. 4). Both treatments were associated with high variation in the disease score between individuals (Fig. 5B), arguing against using NLCs for this drug. RIF was more active against the infection when administered within PLGA or NLC NPs than in the free form, thus demonstrating the strength of the NP approach; of note, the concentrations of RIF used in NPs were about 1000 times lower than those of the free drug in the immersion baths.

Our study provides proof of principle that the zebrafish model is powerful for initial testing of drug formulations against pathogens, providing a selection of effective treatments. These treatments can subsequently be tested in aquaculture fish, although the current study is not meant to be implemented into immediate routine use at aquaculture settings. Since the optimal temperature of zebrafish is 28°C, this model is best suited for testing pathogens of warm water aquaculture fish species such as sea bass and tilapia. Nevertheless, zebrafish can also be cold-adapted to investigate infections of cold water fish such as salmon and cod. Brudal et al. (2014) cold-adapted zebrafish embryos down to 20°C to infect them with *F. naotunensis naotunensis*, a pathogen that devastated the Norwegian Atlantic cod industry (Nylund et al. 2006). Zebrafish adults can be kept at temperature as low as 15°C as shown by an elegant study wherein a promising vaccine system was developed against the viral hemorrhagic septicemia virus (Novoa et al. 2006).

In addition to the attraction of the zebrafish embryo system for evaluating both bacterial infection and the *in vivo* fate of NPs, or the efficacy of drug treatment

via bath immersion, the combination of monitoring embryo survival, bacterial fluorescence, and the multi-parameter disease score provides a powerful, yet simple, set of tools for evaluating drug therapy against an important infectious bacterium of aquaculture-relevant fish.

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