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Nanoliposomes encapsulating immunostimulants modulate the innate immune system and elicit protection in zebrafish larvae

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ABSTRACT

Here we present immunostimulant-loaded nanoliposomes (NL_c) as a strategy to protect zebrafish larvae against bacterial infection. The NL_c encapsulate crude lipopolysaccharide (LPS) from *E. coli* and polyinosinic:polycytidylic acid (Poly I:C), a synthetic analogue of viral dsRNA. Fluorescently-labeled NL_c were ingested by zebrafish larvae 4 days post fertilization, when administrated by bath immersion, and accumulated in the intestine. RT-qPCR analysis showed the expression of innate immune related genes (*tnfα*, *il1β*, *nos2a, irf1a* and *ptgs2a*) was significantly upregulated at 48 h post NL_c treatment. A zebrafish larvae infection model for *Aeromonas hydrophila* was set up by bath immersion, achieving bacterial-dose-dependent significant differences in survival at day 5 post infection in both injured and non-injured larvae. Using this model, NL_c protected non-injured zebrafish larvae against an *A. hydrophila* lethal infection. In contrast, neither the empty nanoliposomes nor the mixture of immunostimulants could protect larvae against lethal challenges. Our results demonstrate that nanoliposomes could be further developed as an efficient carrier, widening the scope for delivery of other immunostimulants in aquaculture.

1. Introduction

Since the late 1980s, the aquaculture industry has grown strikingly to meet the demand of fish for human consumption, when fish capture production reached a plateau. Globally, aquaculture provided 47% of total fish production in 2016, and this trend is still increasing [1]. To develop sustainable aquaculture, it is extremely important to avoid disease outbreaks by implementing preventive immunostimulation or vaccination strategies [2]. In particular, specific treatments against pathogens that infect larvae are needed to avoid disease outbreaks. Fish larvae only have a functional innate immune system at the time of hatching as their adaptive immune responses are not yet well established [3]. Fish larvae are an easy target for pathogens, resulting in huge amount of infections and mortality in aquaculture. Numerous pathogenic bacteria have been identified in fish larvae including those from fresh and marine water species [4–8]. The main symptoms observed in infected larvae are poor growth of individuals, a sudden decrease in survival and malformations [3]. Some antibiotics provide a broad-spectrum antimicrobial activity, but their frequent use has led to a surge in resistant strains. This is an increasing problem for veterinary treatment in aquaculture and even a matter for human health [9]. Antibiotics and other drugs are not effective in fish against viral diseases, though they may be used to control secondary bacterial infections. Viral diseases in larvae or juvenile fish also cause high mortalities and huge economic losses. Significant reported viruses in fish larvae include a picorna-like virus in sea bass [10], herpesvirus in Japanese flounder [11], infectious pancreatic necrosis virus (IPNV) in salmonids [12] and viral nervous necrosis virus (VNNV) in barramundi [13]. Recently, trained immunity has been proposed as an important mechanism for protection, based on epigenetic reprogramming of innate immune cells (for a review see Ref. [14]). Natural killer (NK) cells and macrophages have been shown to exhibit memory-like properties against viruses or against bacteria and fungi, respectively [15–17]. In fish, few prophylactic strategies have been designed for larvae although promising protection has been reported both against viral [18,19] and bacterial infections [20,21]. There is an urgent need to develop suitable, efficient prophylactic tools for fish larvae and to find novel adjuvants to increase vaccine efficacy for both juveniles and adults, especially for those vaccines based on recombinant antigens or inactivated pathogens.

Nanoparticles have been widely used as carriers or delivery systems to enhance the performance of immunostimulants or prolong release of

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vaccines in aquaculture (reviewed in Ref. [22]). Nanoparticles have unique physical characteristics in size, shape, surface chemistry, or targeted surface ligand/receptor that make them highly versatile for drug delivery. The benefits of nanoparticles as delivery tools include facilitate the cellular uptake and presentation of antigens, reduce the doses and improve tissue specific targeting, lessen the toxic or secondary effects of the drug and increase in the delivery effectiveness [23,24]. Liposomes have been shown to be promising vaccine and/or adjuvant carriers aimed to potentiate immune responses through the induction of antibody and T lymphocyte responses with associated subunit antigens [25]. They have a hydrophilic core, and an exterior, self-sealing, hydrophobic bilayer, allowing encapsulation of both lipophilic and hydrophilic compounds [26]. They provide the potential for greater solubility, enhanced bioavailability, improved controlled release and better precision targeting of the encapsulated material [27]. Liposomes have been used as a carrier, delivering bacterial or viral antigens to adult fish species achieving promising results [28–33]. However, using liposomes for the delivery of immunostimulants in fish larvae are poorly studied (see review [22]). Thus, developing nanoliposomes encapsulating immunostimulants/antigens would be of great interest in the prophylaxis of fish larvae diseases.

In previous work, we developed nanoliposomes (NL_c) encapsulating two immunostimulants: bacterial lipopolysaccharide (LPS) and a synthetic analogue of dsRNA viruses (Poly I:C) and evaluated its behavior in adult zebrafish [34]. The NL_c protected adult zebrafish against otherwise lethal bacterial (*Pseudomonas aeruginosa* PAO1) and viral (spring viraemia of carp virus) infections regardless of whether they were administered by injection or immersion [35]. Zebrafish (*Danio rerio*) has been widely used as a model organism for aquaculture purposes for testing prophylactic approaches adapted to different life cycle stages [36]. We chose zebrafish larvae for the *in vivo* assays for multiple reasons: the innate immune system is active by the first day of embryogenesis [37] and the body is transparent at the early stage or can be depigmented using 1-phenyl 2-thiourea (PTU) allowing real-time visualization [38], they are easy to breed and handle [39] and there is a rising number of markers for immune cells and transgenic lines, allowing further identification of the cell types involved in the immunisation process [40,41]. Here, we studied the biodistribution and the immune-stimulating capability of LPS/PolyI:C nanoliposomes in zebrafish larvae. We also developed an *A. hydrophila* infection model using zebrafish larvae according to Saraceni et al. [42]. We tested NL_c to evaluate whether they could protect zebrafish larvae against this bacterial lethal infection.

2. Materials and methods

2.1. Zebrafish husbandry

Wild type (wt) zebrafish (*D. rerio*) were housed in an aquarium system with a photoperiod of 14 h light/10 h dark at 28 °C. One week before breeding, adult zebrafish (approximately 5 months old) were separated by gender and fed with a rich supply of food (blood worm or brine shrimp). Embryos were obtained by a natural spawning method. Briefly, one female and three males were placed separately in a 3 L spawning tank with a divider in the late afternoon and the fish were put together the next morning to trigger spawning. Embryos were collected and reared in 90 × 14 mm Petri dish (Deltalab) with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 0.1% methylene blue) until hatching. Debris and chorions were removed using a Pasteur pipette (Deltalab) 3 times a day. All experimental procedures involving zebrafish (*Danio rerio*) were authorized by the Ethics Committee of the Universitat Autònoma de Barcelona (UAB, CEEH number 1582) who follow the International Guiding Principles for Research Involving Animals (EU 2010/63).

2.2. Preparation and lyophilization of fluorescent NL_c

The nanoliposomes (NL_c) encapsulating LPS (L2630, Sigma-Aldrich) and Poly I:C (InvivoGen) were prepared as previously described in Ruyra et al. [34]. Liposomes were prepared by the thin film hydration method [43] with some modifications. Briefly, 1,2-didodecanoyl-sn-glycero-3-phosphocholine, cholesterol, cholesteryl and cholesterol-PEG600 were dissolved in chloroform solutions (100 mg/ml) and mixed at the desired molar ratios (0.5:0.35:0.1:0.05), which we refer to as the lipid mixture. To obtain fluorescent liposomes, fluorescein-DHPE (Life technologies) was mixed into the lipid mixture (at 0.01 M ratio). The organic solvent was then evaporated by rotary evaporation to obtain a lipid film (fluorescent nanoliposomes, NL_s). The loaded fluorescent NL_c was made by hydrating the dry lipid film with 1.0 mg/ml LPS and 0.5 mg/ml Poly I:C (immunostimulants) in phosphate buffered saline (PBS) as described previously [35]. The resulting lipid suspensions were then vigorously shaken and homogenized by means of an extruder (Lipex Biomembranes) through 2 stacked polycarbonate membranes (200 nm pore size, Avanti Polar Lipids) to finally obtain unilamellar liposomes. Then, non-encapsulated immunostimulants were removed from the liposome preparation by ultracentrifugation at 110,000 × *g* for 30 min at 10 °C. For long term conservation, the cryoprotectant trehalose was incorporated into the liposomes. The dry lipid film was hydrated with a solution containing the immunostimulants and trehalose at a lipid/carbohydrate ratio of 1:5 (2.7%, w/v). The resulting NL_s and NL_c were frozen in liquid nitrogen, lyophilized for 48 h at −80 °C and stored at 4 °C. When needed, the lyophilized samples were re-suspended in PBS, the particle size distribution and zeta potential (ζ) were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments) and the morphology was examined by Cryo-TEM (JEOL-JEM1400, Japan) before use in the experiments.

2.3. Bath immersion of fluorescent NL_c in zebrafish larvae

The *in vivo* biodistribution of the fluorescent NL_c in zebrafish larvae were studied using bath immersion administration. Zebrafish larvae at 2 days post fertilization (dpf) were treated with fluorescent NL_c at concentrations of 0.75, 1.0 and 1.5 mg/ml prepared in E3 medium. E3 only and E3 containing 10% PBS groups were used as controls. At 24 and 48 h post immersion, larvae were anaesthetized with 160 mg/l MS-222 and examined using a fluorescent microscope (Nikon Eclipse 80i). Images were prepared using Fiji software [44].

2.4. RNA extraction and cDNA synthesis

Zebrafish larvae (2 dpf) were immersed with either 10% PBS, a mixture of 25 μg/ml LPS and 50 μg/ml Poly I:C, fluorescent NL_s (0.75, 1.0 and 1.5 mg/ml) or fluorescent NL_c (0.75, 1.0 and 1.5 mg/ml) in E3 medium. At 48 h post immersion, 20 larvae from each group were sampled for RNA extraction. Total RNA was extracted from the pooled larvae homogenized by a polytron homogenizer (Kinematica) using the Maxwell RSC simplyRNA Tissue Kit (Promega) according to the manufacturer's instructions. The RNA concentration was determined with Nanodrop 1000 (Thermo scientific) and the quality was assessed using the Agilent 2100 Bioanalyzer system (Agilent Technologies). Then cDNA (0.5 μg) was synthesized using the iScript cDNA synthesis kit (Bio-Rad).

2.5. Analysis of immune gene expression by RT-qPCR

RT-qPCR was performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using the iTaq universal SYBR green supermix kit (Bio-Rad) following manufacturer's instructions. In brief, each PCR mixture consisted of 5 μl SYBR green supermix, 0.5 μM specific primers (Table 1), 2.5 μl diluted cDNA and 1.5 μl water (Sigma-Aldrich) in a final volume of 10 μl. Elongation factor 1 alpha (*eef1α1*)

Table 1
Primers for RT-qPCR analysis.

Gene name	Primers	Accession n°
eef1α1	Forward: CTTCTCAGGCTGACTGTGC Reverse: CCGCTAGCATTACCCTCC	AY422992
tnfα	Forward: TGCTTCACGCTCCATAAGACC Reverse: CAAGCCACCTGAAGAAAAGG	NM_212859.2
nos2a	Forward: GAGCAGGCCCAATGCATTT Reverse: TGCGCTGCTGCCAGAAAC	NM_001104937.1
il1β	Forward: CATCAAACCCCAATCCACAG Reverse: CACCACGTTCACTTCACGCT	NM_212844.1
irf1a	Forward: GAGACACGGCTGGAACATCG Reverse: ACCCTGAAGGCGTTGTGGC	NM_001040352.1
ptgs2a	Forward: ACACATGGCATCCGCAACAT Reverse: TGGGCAGCCAGATCTTTGTC	NM_153657.1
gig2e	Forward: AGGGTACGACACTGCCTGGT Reverse: AGGGTCACCAAAGCCACAAT	NM_001245991.1

was used as a reference gene [45]. Dilutions 10^{-2} and 10^{-1} of cDNA were used for amplification of the *eef1α1* gene and immune-related genes, respectively. All the samples were from 3 independent experiments. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [46].

2.6. A. hydrophila culture and zebrafish larvae infection

The *A. hydrophila* strain (AH-1) was provided by Dr. Juan Tomás from the University of Barcelona. For experimental infection, bacteria were grown on LB agar plates overnight at 28 °C. The bacteria were collected from the plates in an Eppendorf tube, washed with PBS and finally resuspended with PBS to obtain a stock solution containing approximately 10^{10} colony-forming units (CFUs)/ml (OD620 nm = 1.3). Dilutions at the desired concentration were all prepared from the stock solution. The *A. hydrophila* infection was carried out by bath immersion using injured and non-injured larvae following the protocol of Saraceni et al. [42] with some modifications. Briefly, injured and non-injured larvae at 5 dpf were prepared for infections. To obtain injured larvae, animals were anesthetized and placed on a Petri dish. A small transection of the tail fin was done using a sterile surgical blade (Albion) under a stereomicroscope (Nikon). Groups of 12 injured and non-injured larvae were distributed on 96-well plates (ThermoFisher) with one larva per well containing 200 µl E3 medium or diluted bacterial

A

Name	Composition	Charge	ξ potential (mV)	Size (nm)
NL_s	Liposome	+	9.56±1.0	216.6±2.9
NL_c	Liposome+LPS+poly I:C	+/-	-1.1±1.9	235.4±1.1

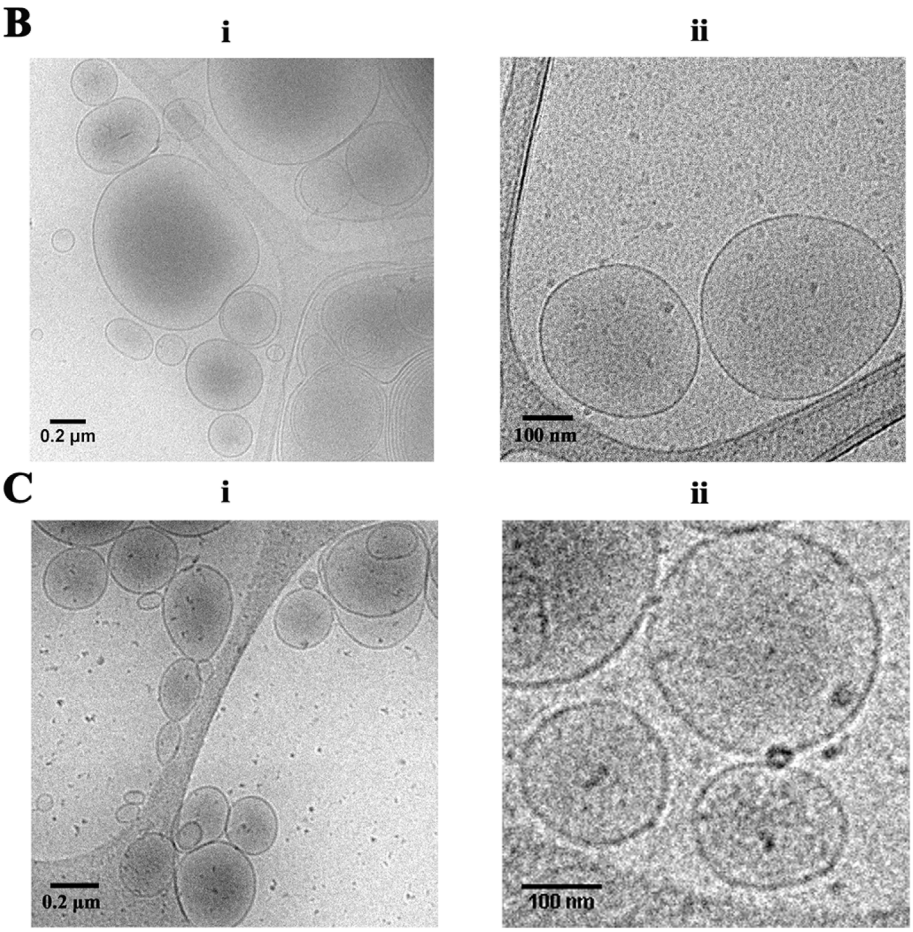

Fig. 1. Characterization of lyophilized NL_c and NL_s liposomes. (A) DLS analysis of LPS/Poly I:C loaded (NL_c) and non-loaded nanoliposomes (NL_s). (B) Representative Cryo-TEM image of lyophilized NL_c after re-hydration with PBS (i) and the image with a higher magnification (ii). (C) Representative Cryo-TEM image of lyophilized NL_s after re-hydration with PBS (i) and the image with a higher magnification (ii).

suspensions. For infection, the bacteria were diluted from the stock solution using E3 medium in serial 100-fold dilutions from 10^{-2} to 10^{-6}. The bacteria dilutions were kept in each well throughout the whole experiment at 28 °C. Injured and non-injured larvae immersed in 10% (v/v) PBS in E3 medium were used as controls. The bacterial dilutions of 10^{-7} and 10^{-8} (100 µl of each) from stock solution were inoculated on LB plates and incubated overnight at 28 °C to calculate the real CFUs during the infection. The survival curves were analyzed and statistical differences were assessed using the log-rank test (GraphPad Prism v7.0).

2.7. *A. hydrophila* challenge of zebrafish larvae after NL_c administration

Zebrafish larvae (2 dpf) were immersed in either 10% PBS, a mixture of 12.5 µg/ml LPS and 25 µg/ml Poly I:C, 0.75 mg/ml NL_s or 0.75 mg/ml NL_c prepared in E3 medium for 48 h. The immersion experiments were performed in 6 well plates with 50 larvae per group. At 48 h post immersion, the larvae were washed with E3 medium and the uptake of nanoliposomes was confirmed by observing the larvae under the fluorescent microscope. The larvae were kept in E3 medium for an additional 24 h. At 5 dpf, the larvae were challenged with *A. hydrophila* (around 10^8) and survival was monitored during the following 5 days. *A. hydrophila* was inoculated overnight on a LB plate one day before the infection experiments. The actual CFUs for infection were counted by inoculating 100 µl of diluted 10^{-7} and 10^{-8} bacterial suspension on LB plates overnight at 28 °C. All the experiments were done in triplicate and 24 larvae/group were used in the infection experiments. A Kaplan-Meier survival plot was generated and statistical differences were evaluated using the log-rank test (GraphPad Prism v7.0).

3. Results

3.1. Characterization of lyophilized nanoliposomes

To characterize the physicochemical structure of the lyophilized liposomes after long-term storage at 4 °C, NL_s and NL_c were assessed by dynamic light scattering (DLS) and Cryo-TEM. The DLS result showed that the average size of both lyophilized NL_s and NL_c (Fig. 1A) are slightly bigger than those freshly prepared (182.7 ± 8.4 nm and 125.8 ± 6.6 nm, respectively) as described previously [34,35]. In contrast, the ξ potential values of both NL_s and NL_c were maintained as positive and neutral (Fig. 1A), respectively. The Cryo-TEM showed that the morphology of NL_s and NL_c were mostly maintained as small unilamellar vesicles (Fig. 1B and C). A proportion of NL_s and NL_c were multilamellar vesicles and were slightly larger than the unilamellar vesicles. These results were confirmed with DLS data showing that the monodisperse size distribution of liposomes is slightly larger and has an average diameter of around 200 nm.

3.2. Fluorescent NL_c were ingested by zebrafish larvae

Zebrafish larvae (2 dpf) were immersed into three different concentrations of NL_s and NL_c for 24 and 48 h. E3 medium and 10% PBS treated larvae were used as controls. Fluorescent microscopy images showed that NL_s and NL_c are mainly found in the intestine of zebrafish larvae at 48 h post immersion (Fig. 2). Interestingly, some NL_c were also found in the pharynx between the eyes and yolk (Fig. 2B: NL_c 1.5 mg/ml). No fluorescence was observed in the PBS treated and non-treated larvae after 48 h of immersion. Moreover, no fluorescence was observed in the larvae treated with either NL_s or NL_c at 24 h post immersion (data not shown). These results suggest that the nanoliposomes might be ingested by larvae through the oral system and then accumulated into the digestive system.

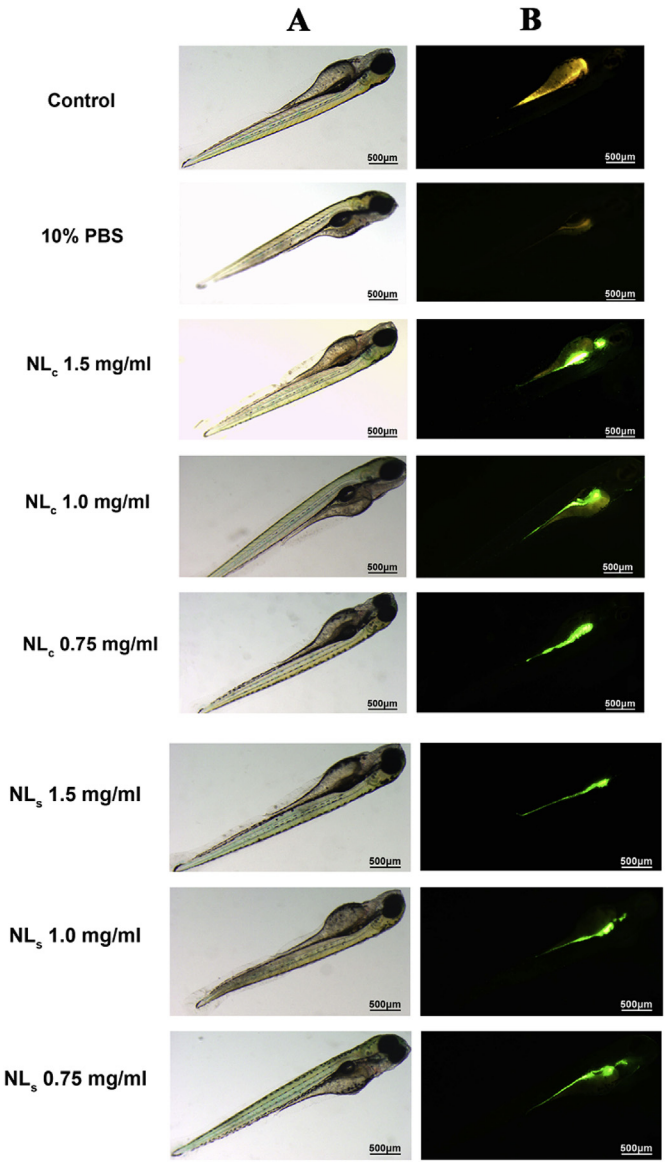

Fig. 2. Biodistribution of fluorescent NL_c and NL_s in zebrafish larvae. Zebrafish larvae (2 dpf) were immersed in 0.75, 1.0 and 1.5 mg/ml of both NL_c and NL_s for 48 h. E3 water and 10% PBS immersed larvae were used as controls. (A) Representative light transmission images. (B) Representative fluorescent images.

3.3. NL_c up-regulate the expression of immune-related genes

Zebrafish larvae (2 dpf) were immersed either in PBS (control), a mixture of LPS and Poly I:C (the same amount of immunostimulants encapsulated in the 1.5 mg/ml NL_c), and three concentrations of both NL_s and NL_c for 48 h. We observed a significantly higher expression of the *tnfα* and *il1β* gene in the 0.75, 1.0 and 1.5 mg/ml NL_c groups (Fig. 3). The expression of *nos2a* showed a very high fold-change pattern in 0.75 and 1.0 mg/ml NL_c groups and a significant higher fold change in 1.5 mg/ml NL_c group than the control. The means of fold-changes were ~165, 125 and 30 in the 0.75, 1.0 and 1.5 mg/ml NL_c groups, respectively. The expression of *irf1a* was significantly higher in all the NL_s and NL_c groups compared to the control with an approximately 20 fold-changes except in 0.75 mg/ml NL_c group. In addition, we also examined the pro-inflammatory (*ptgs2a*) and anti-viral genes (*gig2e*) and the expression of these two genes was slightly upregulated (Fig. 3).

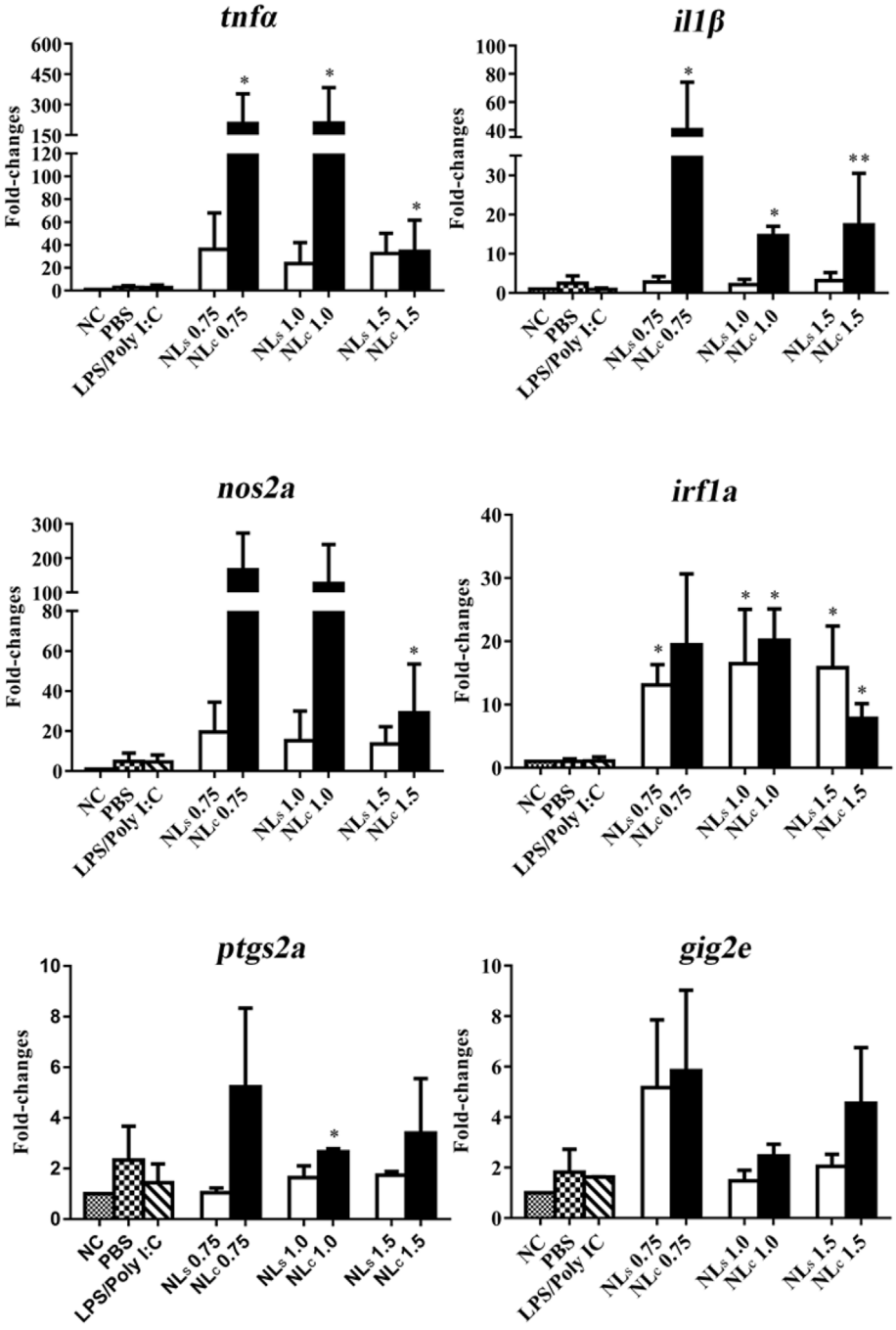

Fig. 3. Immune-related gene expression analysis by RT-qPCR. Zebrafish larvae (2 dpf; n = 25 larvae) were immersed in PBS, a mixture of free LPS and Poly I:C, NL_s (0.75, 1.0 and 1.5 mg/ml) and NL_c (0.75, 1.0 and 1.5 mg/ml) for 48 h. The gene expression levels of pooled samples are shown as fold change using the Livak method ($2^{-\Delta\Delta Ct}$). Statistical significance used a one-way unpaired *t*-test to compare each gene's mean C_T values with control using Welch's correction for unequal variances (GraphPad). Results are from 3 independent experiments.

3.4. A. hydrophila infection model in zebrafish larvae

To test whether the nanoliposomes could protect larvae against bacterial infection, we firstly set up the *A. hydrophila* infection in larvae by immersion administration. We used both injured and non-injured larvae (5 dpf) and three doses of bacterial suspension (10^4, 10^6 and 10^8 cfu/ml). The mortality was calculated within 5 days after infection. The survival curves are shown in Fig. 4A and B. In the non-injured larvae experiments, the survival was significantly lower in the 10^8 cfu/ml infection group than the control. No significant differences were found in the 10^6 and 10^4 cfu/ml infection groups compared to the control. In injured larvae experiments, the mortality was significantly higher in the 10^8 cfu/ml infection group than the control. No significant differences were found in the 10^6 and 10^4 cfu/ml infection groups compared to the control. However, the variabilities of the three biological replicates (standard deviation of the means) were much higher in the injured group than in the non-injured group of all three infection doses. The relative higher standard deviation in injured group may due to the variable real infection doses among the experiments and the additional manipulation of the larvae. Therefore, we used the non-injured larvae for our further experiments to minimize the handling of larvae and to obtain reliable results.

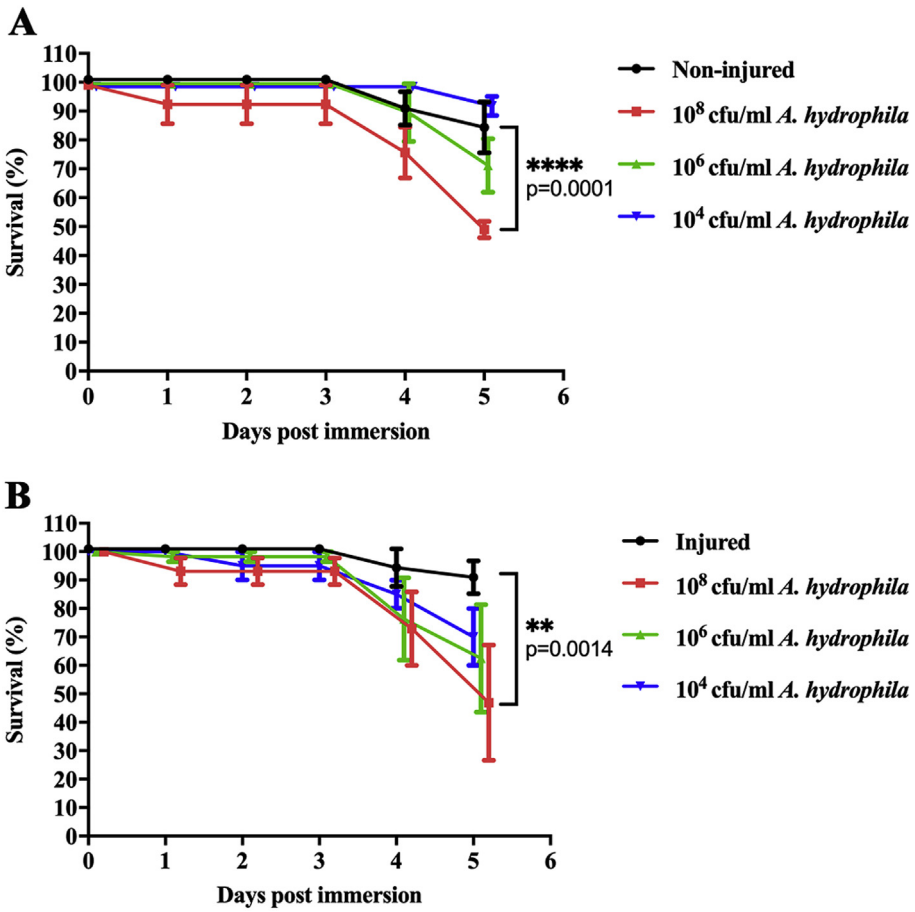

Fig. 4. Zebrafish larvae *A. hydrophila* infection. Zebrafish larvae (5 dpf; n = 12) were infected with *A. hydrophila* by bath immersion. Survival was recorded every 24 h for 5 days. (A) Non-injured larvae infection with $(1.2 \pm 0.7) \times 10^8$, $(1.2 \pm 0.7) \times 10^6$ and $(1.2 \pm 0.7) \times 10^4$ cfu/ml *A. hydrophila*. (B) Injured larvae infection with $(1.2 \pm 0.7) \times 10^8$, $(1.2 \pm 0.7) \times 10^6$ and $(1.2 \pm 0.7) \times 10^4$ cfu/ml *A. hydrophila*. The results show the mean ± SD of 3 independent experiments and the differences were analyzed using Two-way ANOVA post Dunnett test (**, $p<0.01$; ****, $p<0.0001$).

3.5. NL_c protect zebrafish larvae against A. hydrophila lethal challenge

Having confirmed that zebrafish larvae at 4 dpf could ingest NL_c and larvae at 5 dpf could be infected with *A. hydrophila,* both being administered by immersion, we evaluated the NL_c protection to zebrafish larvae against this bacterial lethal challenge. The RT-qPCR analysis showed that the lowest dose (0.75 mg/ml) exhibits highest fold changes among 0.75, 1.0 and 1.5 mg/ml groups and we did not observe any toxicity at 0.75 mg/ml (data not shown). Thus, we chose the dose of 0.75 mg/ml for the immunostimulation of larvae. Larvae at 2 dpf were immersed with 0.75 mg/ml NL_c for 48 h. After the confirmation of ingestion at 4 dpf, the larvae at 5 dpf were challenged with 10^8 cfu/ml *A. hydrophila*. The mortality was calculated within the next 5 days after infection and a representative survival curve is shown in Fig. 5. The result showed that the larvae survival was significantly higher in the NL_c treated group than in the non-treated group. In contrast, no significant differences were found in either free LPS and Poly I:C treatments or in NL_s treated groups compared to the non-treated control.

4. Discussion

Millions of microorganisms are present in the aquatic environment and are especially threatening to fish during the early developmental stages when the immune system is still immature. Although a mixed passive immunity exists during vitellogenesis and oogenesis [47,48], fish larvae survival depend fundamentally on their innate defense mechanisms, since adaptive immunity develops later. An active innate immune system is detectable in zebrafish larvae at 1 dpf whereas a functionally mature adaptive immune system is not active until 3 weeks post fertilization [37,49]. This different maturation time allows us studying innate immune responses to immunostimulation or/and infection independently from the adaptive immune system. Also in aquacultured fish species such as gilthead sea bream, most of the innate

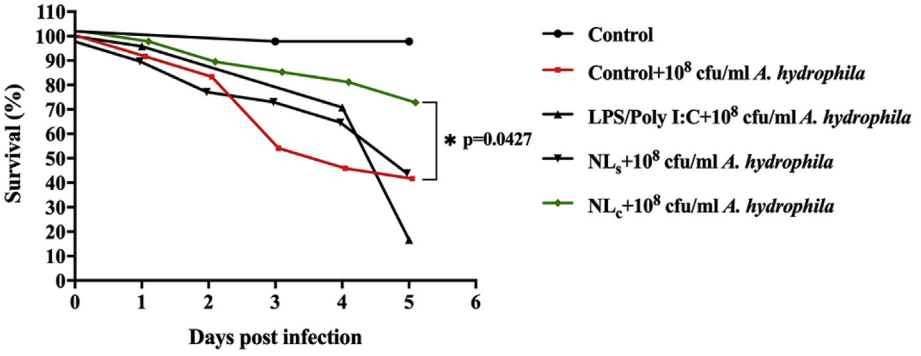

Fig. 5. Representative survival of the treated zebrafish larvae challenged with *A. hydrophila*. Zebrafish larvae (2 dpf; n = 24) were immersed in NL_c, NL_s, a mixture of LPS and Poly I:C and PBS for 48 h. After 24 h, the larvae (5 dpf) were challenged with *A. hydrophila* at the dose of $(1.1 \pm 0.3) \times 10^8$ cfu/ml. Larvae immersed with PBS followed by *A. hydrophila* challenge was used as a mortality control. Cumulative survival curves were constructed using GraphPad. Significant differences were analyzed using Log-rank (Mantel-Cox) Test. *$p<0.05$.

immune genes are already expressed at hatching, including those coding for innate immune receptors (*tlr5* and *tlr9*), pro-inflammatory (*il-1β*, *tnfα* and *cox2*) and anti-inflammatory (*tgf-β1*) molecules, anti-viral (*mx*, *irf1*, *irf-9*, *ifi-30* and *prt-1*) and antibacterial (*c3*, *lyz*, *hamp* and *lbp/bpi*) molecules and phagocyte markers (*m-csfr* and *ncf4*) [50]. It had been proposed that the immunomodulation of fish larvae could be a potential method for improving larvae survival by increasing the innate responses [50]. In other words, the immune defenses of fish larvae against pathogens could be enhanced by stimulating the innate immune system.

After an extensive characterization of the response to NL_c in adult zebrafish [35], we evaluated NL_c as an immunostimulants carrier in larvae. Firstly, we confirmed that the nanoliposomes remained intact after the lyophilization procedure allowing long-term conservation (up to two years). The stability of vaccines and adjuvants during storage and distribution is a critical issue for potential commercialization, regardless of how effective a vaccine is in the laboratory [51]. Next, we showed that the fluorescent NL_c were ingested by zebrafish larvae through bath immersion. We focused on bath immersion because is the only convenient method for immunostimulation of fish larvae due to their small size and fragility when handled. In addition, due to larvae's intrinsic vulnerability any prophylactic approach should be non-toxic. A degree of toxicity of cationic liposomes has been described previously, caused by their cationic nature [34,52]. We did not observe any toxic effects using NL_c cationic liposomes at the doses tested but a certain level of toxicity has been observed at higher doses (more than 1.5 mg/ml, data not shown). After immersion, the NL_c nanoliposomes were found in the pharynx and intestine of 4 dpf larvae but not of 3 dpf larvae. This is probably because the mouth of the zebrafish larvae is not widely open until 3 dpf [53]. Nikapitiya et al., had shown that chitosan nanoparticles were ingested by zebrafish larvae and stimulated the expression of several innate immune genes [54]. Successful absorption of immunostimulants or antigens to the intestine could be very important to develop strong mucosal and systemic responses against a later infection through natural routes.

In adults, the main immune tissue involved in pathogen recognition is the gut-associated lymphoid tissue (GALT) which has a diffuse organization and contains lymphoid cells, macrophages, eosinophils and neutrophils [55]. In larvae there is scarce information about the structure and functionality of GALT since its ontogeny is not well understood. However, some works point out the importance of this tissue in the early recognition of pathogens [56] and for example in meagre, GALT is already present at 12 dph [57]. At present no information about the ontogeny of GALT in zebrafish larvae is available but IgZ gene expression has been reported at initial stages of embryonic development (6 dpf) [58]. The innate immune system of zebrafish larvae is functional since 1 dpf [59]. The activation of the immune system is highly dependent on whether the immune cells could recognize the pathogens/immunostimulants as potential risk molecules. Mature neutrophils and macrophages are present at 24–30 hpf [60], which are the primary actors in the larvae innate immune system [61].

To understand the effect of NL_c on the innate immunity of zebrafish larvae, we examined the differential expression of certain immune genes. The selection was made according to their different functions in the innate immune system and to the fact that these genes are already expressed as mentioned in Ref. [62]. *Tnfα* and *il1β* are key markers of proinflammatory and antibacterial activity [63], mainly produced by activated macrophages in different immune activation pathways [64]. *Nos2a* is an early feature of inflammation expressed in macrophages and other phagocytes [65]. *Irf1a* and *gig2e* are markers of the immune response to viral infection [66–68]. *Ptgs2a* is an inflammatory related enzyme and its expression can be induced by LPS [69]. The up-regulation of the immune-related genes *tnfα*, *il1β, nos2a* and *irf1a* indicates a strong stimulation of the zebrafish larvae immune system by NL_c. However, other genes, namely *ptgs2a* and *gig2e*, showed a slight up-regulation. These differences in gene expression could be due to the highly tolerogenic environment of the intestine under development [70]. It may also explain that the changes of some genes were variable among triplicates, such as *tnfα* and *nos2a* genes. It is worth mentioning that similar results were also found in our previous studies *in vitro* in which NL_c elicited variable pro-inflammatory and anti-viral responses in zebrafish hepatocytes [34]. Such variable effects of liposomes encapsulating immuostimulants on the modulation of immune responses need to be further studied.

To see whether the successful uptake and immune stimulation provided by NL_c could indeed protect fish larvae from a bacterial infection, we investigated the survival rate of challenged zebrafish larvae against *A. hydrophila* administrated by bath immersion. The zebrafish larvae infection model with *A. hydrophila* at 4 dpf had been established by micro-injection administration and bath immersion [42]. We chose bath immersion route because it is a suitable and convenient administration method for both immunostimulation and challenge in fish larvae. It is less labor-intensive and mimics the natural exposure to infection. We modified the protocol from Saraceni et al. [42] to be suitable for testing the immunostimulation by NL_c followed by *A. hydrophila* infection. The 5 dpf larvae infected with *A. hydrophila* showed significantly higher mortality in both injured and non-injured groups. The data also showed a high variability of survival in triplicates. Nakanishi et al. pointed out that antigen uptake was highly influenced by the duration of exposure, total biomass, age, pH, and salinity of the water used for immersion and the uptake of antigen among individuals is variable comparing to injection route [71]. We used the non-injured infection method for further experiments due to the easier manipulation of infection and the smaller deviation in the mortality. The same approach was used to test immunomodulation and survival after chitosan nanoparticle treatment and *A. hydrophila* infection [54]. We achieved consistent high survival in NL_c group comparing to the controls (Fig. 5) and thus we demonstrated that NL_c protected zebrafish larvae against an *A. hydrophila* lethal infection.

Summarizing, here we present a novel system based on liposomes encapsulating immunostimulants suitable to protect fish larvae. Nanoliposomes could encapsulate not only immunostimulants but also could be further modified to enclose vaccines or fragile antigens for both the larvae and adult stage. We show that NL_c could be ingested by zebrafish larvae by bath immersion and stimulate the innate immune system involved in anti-viral and anti-bacterial responses. We also show that NL_c protected zebrafish larvae against *A. hydrophila* infection. Accordingly, we propose that a nanoliposomes encapsulation strategy could be incorporated in aquaculture for the delivery of immunostimulants or antigens to reduce susceptibility to diseases, especially in fish larvae. Future studies should focus on investigating the use of nanoliposomes to deliver other immunostimulants or vaccines, and should look into cost-effectiveness, efficacy and long-term protection.

Authorship

NR and JJ designed the study. JJ performed all the experiments. SM and JTM provided the *A. hydrophila* strain and helped with the *A. hydrophila* infection model. JJ and NR wrote the manuscript and prepared all the tables and figures. All authors contributed to the preparation of the final manuscript.

Declarations of interest

None.

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