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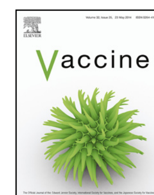

Targeting and stimulation of the zebrafish (*Danio rerio*) innate immune system with LPS/dsRNA-loaded nanoliposomes

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ABSTRACT

Herein we report the use of immunostimulant-loaded nanoliposomes (called *NL_c liposomes*) as a strategy to protect fish against bacterial and/or viral infections. This work entailed developing a method for *in vivo* tracking of the liposomes administered to adult zebrafish that enables evaluation of their *in vivo* dynamics and characterisation of their tissue distribution. The NL_c liposomes, which co-encapsulate poly(I:C) and LPS, accumulate in immune tissues and in immunologically relevant cells such as macrophages, as has been assessed in trout primary cell cultures. They protect zebrafish against otherwise lethal bacterial (*Pseudomonas aeruginosa PAO1*) and viral (*Spring Viraemia of Carp Virus*) infections regardless of whether they are administered by injection or by immersion, as demonstrated in a series of *in vivo* infection experiments with adult zebrafish. Importantly, protection was not achieved in fish that had been treated with empty liposomes or with a mixture of the free immunostimulants. Our findings indicate that stimulation of the innate immune system with co-encapsulated immunostimulants in nano-liposomes is a promising strategy to simultaneously improve the levels of protection against bacterial and viral infections in fish.

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1. Introduction

The immune system of vertebrates encompasses adaptive immunity and innate immunity, the former of which involves immunological memory. Fish posses a highly diverse, strong innate immune system and were the first vertebrates to develop an adaptive immune system. Interestingly, fish lack IgG and class switch-recombination machinery [1], but have IgM, IgT and IgD generated by somatic rearrangement, somatic mutation and gene conversion [2]. Another important distinctive feature of teleosts is that they have phagocytic B lymphocytes. It has been reported the presence of phagocytic B lymphocytes in trout, catfish, cod and Atlantic salmon ([1] and references herein) but not in zebrafish [3]. Nevertheless, farm-raised fish respond well to vaccination. Recently, the concept of "innate memory" has been proposed [4,5] and has also inspired the design of vaccination approaches focused on the stimulation of innate immunity.

Several fish vaccines against viral or bacterial diseases, most of which comprise inactivated pathogens are now available [6]. However, researchers are working intensively to enhance vaccine efficiency by developing new vaccines, containing adjuvants and immunostimulants [7], and new formulations based on encapsulation [8–12]. Encapsulating vaccines makes them more stable to the environment and to low pH and/or enzymatic reactions inside the treated organism [12,13]. Among the various encapsulation systems available, liposomes are especially attractive, as they are biocompatible and highly tuneable [14]; can actually enhance the efficacy of the vaccine, as has been reported in fish [15,16]; and can be used as labels to enable *in vitro* or *in vivo* tracking of the vaccine. Another factor that researchers are endeavouring to improve in

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fish vaccines is administration, which is typically done by injection in adults. Research efforts are focused on creating non-stressful, easy to manage and low-cost vaccination protocols to improve large-scale procedures based on immersion rather than on injection [6,17].

Our group recently developed nanoliposomes (called *NL$_c$ liposomes*) for simultaneous wide-spectrum anti-bacterial and anti-viral protection of farm-raised fish. First, we co-encapsulate two general immunostimulants: bacterial lipopolysaccharide (LPS) and poly(I:C), a synthetic analogue of dsRNA viruses. Then, we demonstrated that the NL$_c$ liposomes were taken up *in vitro* by macrophages and that they regulated the expression of immunologically relevant genes (likely, by triggering innate immune signalling pathways) [18].

In the work reported here, we studied the biodistribution and immunological efficacy of NL$_c$ liposomes in zebrafish *in vivo*. We chose zebrafish as the model organism for the *in vivo* assays for multiple reasons: they have been widely used to study the pathogenicity of different fish and human pathogens; they have innate and adaptive immune systems; and they are easy to breed and handle [19]. We adapted a non-invasive imaging method widely used in mammalian models [20,21], and then used it to track the nanoliposomes in adult zebrafish *in vivo*. To the best of our knowledge, this is the first report of this method being applied to live zebrafish. In addition, we studied which cells were preferentially targeted by the NL$_c$ liposomes in rainbow trout (*Oncorhynchus mykiss*), by performing *ex vivo* analysis of the main immune relevant tissues. We also developed a new model for infection of adult zebrafish by the bacterium *Pseudomonas aeruginosa*, an opportunistic pathogen in fish and in humans [22,23]. Although most of the zebrafish infection models employ larvae [24], the maturity of larval immune systems remains poorly understood. We believe that the development of infection models in adult zebrafish might ultimately prove valuable for designing new therapeutic approaches and for elucidating the functions of the teleost immune system.

2. Materials and methods

2.1. Preparation and lyophilisation of NL$_c$ liposomes

The NL$_c$ (*NanoLiposome cocktail*) liposomes were prepared as previously described in Ruyra et al. [18]. Liposomal formulations were prepared by the thin film hydratation method [25] with some modifications. Briefly, DOPA, DLPC, cholesterol, cholesteryl and chol-PEG600 were dissolved in chloroform solutions (100 mg/ml) and mixed at the desired molar ratios (0.5:0.35:0.10:0.05). The organic solvent was then evaporated by rotary evaporation to obtain a dry lipid film. For the preparation of the liposomes that contained a cocktail of immunostimulants the dry lipid film was hydrated with a solution containing 0.5 mg/ml poly(I:C) and 1.0 mg/ml LPS in PBS. The co-encapsulation of poly(I:C) and LPS was done with an immunostimulant:lipid ratio of 1:30 and 1:15, respectively. The resulting lipid suspensions were then vigorously shaken and were homogenised by means of an extruder (Lipex Biomembranes, Canada) through 2 stacked polycarbonate membranes (200 nm pore size, Avanti Polar Lipids) to finally obtain unilamellar liposomes. In all cases, non-encapsulated immunostimulants were removed from liposome preparations by ultracentrifugation at $110{,}000 \times g$ for 30 min at 10 °C. Liposome integrity was checked by DLS and Cryo-TEM. The final NL$_c$ liposomes comprised 125.8 ± 6.6 nm liposomes containing both poly(I:C) and LPS (1 mg/ml liposome encapsulates 33.3 μg/ml poly(I:C) and 16.6 μg/ml LPS) and had a neutral surface charge (1.37 ± 3.58 mV). The co-encapsulation efficiencies (EE) were of $22.3 \pm 2.1\%$ for LPS and of $99.6 \pm 0.1\%$ for poly(I:C). For long-term conservation, the cryoprotectant trehalose was incorporated into the procedure. 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$_c$ liposomes were frozen in liquid nitrogen, lyophilised (48 h at −80 °C) and finally, stored at RT for several weeks. When needed, the lyophilised samples were resuspended in PBS and the morphology of the reconstituted NL$_c$ liposomes was assessed by Cryo-TEM (JEOL-JEM 1400, Japan). To quantify the amount of immunostimulants leaked after lyophilisation, liposomes encapsulating either poly(I:C) or LPS were prepared lyophilised and finally, stored at RT. At 0 h and 4 months, the dried liposomal cakes were resuspended with PBS and the free poly(I:C) or LPS was separately quantified as described in Ruyra et al. [18].

2.2. Animals

Adult wild type (wt) zebrafish were held in tanks with recirculating water under 14 h light/10 h dark at 28 °C. Adult rainbow trout (*O. mykiss*) were held in tanks under 12 h light/12 h dark at 15 °C. All the experimental protocols with animals were reviewed and approved by the Ethics and Animal Welfare Committee and Biosecurity Committee of the Universitat Autònoma de Barcelona and Universidad Miguel Hernandez. All of these protocols followed the International Guiding Principles for Research Involving Animals.

2.3. Fluorescent labelling of NL$_c$ liposomes

Alexa Fluor 750 (AF750) succinimidyl ester and DOPE-NH$_2$ were conjugated as previously described [25]. Only conjugated Alexa Fluor 750 was detected by TLC (Rf = 0.6), indicating that conjugation was complete. The fluorescently labelled AF750-NL$_c$ liposomes were prepared by incorporating AF750-DOPE into the lipid mixture (0.01 molar ratio). Similarly, fluorescently labelled FITC-NL$_c$ liposomes were prepared by incorporating Fluorescein-DHPE (Molecular Probes, Life Technologies Corp., USA) into the lipid mixture (0.01 molar ratio).

2.4. Biodistribution of the NL$_c$ liposomes in zebrafish

The *in vivo* biodistribution of the NL$_c$ liposomes in adult zebrafish (0.39 ± 0.04 g weight) was studied using the AF750-NL$_c$ liposomes. The liposomes were administered by intraperitoneal (i.p.) injection or by immersion. Administration by i.p. injection: adult zebrafish ($n = 4$ per condition) were anaesthetised (MS-222, 40 ppm) and given 10 μl of AF750-NL$_c$ liposomes (380 mg/kg liposome containing 12.6 mg/kg of poly[I:C] and 6.3 mg/kg of LPS). At 24, 48 and 72 h post-injection, the fish were anaesthetised (160 ppm) and imaged in the IVIS Spectrum platform (excitation: 745 nm; emission: 800/820/840 nm, Calliper, PerkinElmer, USA). For the *ex vivo* imaging, the zebrafish were killed by over-anaesthetisation (200 ppm) and their organs were extracted and then, imaged in the IVIS Spectrum platform. Administration by immersion: adult zebrafish ($n = 4$ per condition) were immersed in a tank containing AF750-NL$_c$ liposomes (500 μg/ml liposome containing 16.6 μg/ml of poly(I:C) and 8.3 μg/ml of LPS) for 30 min, and then placed back into a tank of clean water. At 0 and 12 h post-immersion, the fish were anaesthetised and imaged in the IVIS Spectrum platform (as described above). For the *ex vivo* imaging analyses, the zebrafish were killed by over-anaesthetisation (200 ppm), and their organs were extracted and then, imaged in the IVIS Spectrum platform. The images were analysed using Caliper Living Image 4.1 software (PerkinElmer). For the *ex vivo* analysis, the Region of Interest (ROI) was measured and the data were represented as the Radiance Efficiency (RE) divided by the mean area of each organ.

2.5. Targeting of cells by the NL_c liposomes in rainbow trout

FITC-NL_c liposomes were used to study the cells targeted by the NL_c liposomes in rainbow trout. Animals ($n = 4$, ~125 g weight) were anaesthetised and i.p. injected with 200 μl of FITC-NL_c liposomes (96.0 mg/kg liposome containing 3.18 mg/kg of poly(I:C) and 1.59 mg/kg of LPS) or 200 μl PBS (controls). After 24 h, the fish were sacrificed for head kidney and spleen dissection. Adherent trout monocyte/macrophages were isolated as previously described [26]. Every 24 h, cells were studied by flow cytometry analysis (FACSCanto cytometer, Becton Dickinson, USA) or by confocal microscopy imaging (Zeiss LSM 700, Germany).

2.6. P. aeruginosa bacterial challenge in zebrafish after NL_c liposome administration

Adult zebrafish (0.61 ± 0.12 g weight) were transferred to an isolated system and acclimated for 1 day before each experiment. *P. aeruginosa* (PAO1, sub-line MPAO1; obtained from Seattle PAO1 transposon mutant library, University of Washington) was grown at 37 °C in blood agar plates (BioMérieux, France), collected directly from the plates and then, dispersed in sterile PBS. The LD_{50} for PAO1 infection was calculated in fish infected by i.p. injection with 20 μl of PAO1 suspension at concentrations ranging from 3.2×10^7 to 2.5×10^8 cfu. The fish were observed daily for signs of disease and mortality, and the dead fish were assessed for bacterial presence and identification (data not shown). For the survival experiments, the fish were i.p. injected with either 10 μl of NL_c liposome (246 mg/kg liposomes containing 8.2 mg/kg poly(I:C) and 4.1 mg/kg LPS), 10 μl of empty liposomes (246 mg/kg), 10 μl of a mixture of the free immunostimulants (8.2 mg/kg poly(I:C) and 4.1 mg/kg LPS) or 10 μl of PBS (control). At 1, 7 or 30 days post-injection (dpi), the fish were challenged with *P. aeruginosa* ($1.5 \times LD_{50}$) and their survival was assessed for 5 days. All experiments were done in triplicate and 12 individuals were used for each condition and experiment. A total number of 36 fish were used for each condition. Survival curves were analysed using the Kaplan–Meier method and the statistic differences were evaluated using the log-rank test (GraphPad, USA). Relative percentage of survival (RPS) was calculated according to RPS (%) = [(1 − mortality treated group)/mortality control] × 100.

2.7. Cell cultures and Spring Viraemia of Carp Virus (SVCV) preparation

The fish-cell line ZF4 [27] used in this work was purchased from the American Type Culture Collection (ATCC number CRL-2050). ZF4 cells were maintained at 28 °C in a 5% CO_2. The 56/70 isolate of SVCV isolated from carp [28] was propagated in ZF4 cells at 22 °C. Supernatants from SVCV-infected cell monolayers were clarified by centrifugation at 4000 × g for 30 min and stored in aliquots at −70 °C. The clarified supernatants were used for *in vivo* infection assays.

2.8. Spring Viraemia of Carp Virus (SVCV) challenge in zebrafish after liposome administration

Zebrafish were given NL_c liposomes, empty liposomes or a mixture of the free immunostimulants by either i.p. injection or immersion, as described below. I.p. injection: the fish were injected with either 10 μl of NL_c liposomes (246 mg/kg liposome containing 8.2 mg/kg poly(I:C) and 4.1 mg/kg LPS), 10 μl of empty liposomes (246 mg/kg), 10 μl of the mixture of free immunostimulants (8.2 mg/kg poly(I:C) and 4.1 mg/kg LPS) or 10 μl of PBS (control). Immersion: the NL_c liposomes (500 μg/ml liposomes containing 16.6 μg/ml poly(I:C) and 8.3 μg/ml LPS), empty liposomes (500 μg/ml) and a mixture of the free immunostimulants (16.6 μg/ml poly(I:C) and 8.3 μg/ml LPS) were each administrated for 30 min, including a handling control. At 7 dpi, the zebrafish ($n = 15$/each condition) were infected by immersion with SVCV ($7.1 \pm 2 \times 10^7$ pfu/ml) according to previously described infection protocols [29,30]. Fish were assessed for survival, abdominal distension, exophthalmia, impaired swimming and skin/fin base haemorrhages for 15 days. Survival curves were analysed using the Kaplan–Meier method and the differences were evaluated using the log-rank test (GraphPad). Relative percentage of survival (RPS) was calculated according to RPS (%) = [(1 − mortality treated group)/mortality control] × 100. At 5 dpi, two surviving fish from each group were randomly sampled for virus recovery [30].

3. Results

3.1. Biodistribution of NL_c liposomes in zebrafish after administration by i.p. injection

The biodistribution of the NL_c liposomes in adult zebrafish was studied following i.p. injection of the fish with fluorescently labelled liposomes (AF750-NL_c liposomes). Whole-animal images revealed a fluorescence signal in the peritoneal cavity of all the individuals up to 72 h with no detectable fluorescence signal in any other part of the fish (Fig. 1A). Quantification of this signal confirmed a sustained presence of the liposomal formulation. A slight decrease was observed at 72 h: from 3.76×10^9 Radiant Efficiency (RE) at 0 h to 2.16×10^9 RE at 72 h (Fig. 1B). Organ *ex vivo* analysis was performed at 0, 24, 48 and 72 h post-injection, and the corresponding signal intensities were quantified (Fig. 1C). Significant accumulation of the NL_c liposomes was observed in the spleen from 0 to 72 h (from 1.92×10^6 RE/organ area at 0 h to 1.05×10^6 RE/organ area at 72 h), and in the liver at 72 h (5.71×10^5 RE/organ area). These values are consistent with those from previous studies using radioactive labelling, which had shown that large unilamellar liposomes injected into fish had localised mainly in the spleen [13].

3.2. Cells targeted by NL_c liposomes in rainbow trout

To identify the cells targeted by the NL_c liposomes *in vivo*, we worked with adult rainbow trout instead of zebrafish, as the larger size of the former enabled us to isolate mononuclear phagocytes from the main immunologically related organs (spleen and head kidney) for subsequent characterisation by flow cytometry and by confocal microscopy. In a typical experiment, fluorescent NL_c liposomes were injected into trout ($n = 4$), and at 24 h post-injection the spleen and the head kidney were dissected for primary cell culture. The NL_c liposomes were tracked by flow cytometry and by confocal microscopy at 24, 48 and 72 h. Fluorescence signals were significantly detected by flow cytometry (Fig. 2A) in spleen-derived cells at 24, 48 and 72 h. NL_c liposomes were also found in head kidney-derived cells, although in far lower levels than in the spleen. For example, at 72 h, the percentage of total positive cells in the spleen was 30.3 ± 12.6%, compared to 2.9 ± 1.2% for the head kidney. Interestingly, fluorescent cells were detected even up to 6 days post-injection, indicating that the NL_c liposomes can persist for at least 1 week (data not shown). For the confocal microscopy analysis, the cell membranes and nuclei were stained with either CellMask or Hoechst, respectively. The monocytes/macrophages were easily distinguishable by the kidney-shaped nuclei and the rugosity of their plasma membranes (Fig. 2B and C) [31,32]. The presence of NL_c liposomes in macrophage-like cells from the spleen was confirmed at 24, 48 and 72 h (Fig. 2B). Fluorescent NL_c liposomes were also found in macrophage-like cells isolated from head kidney

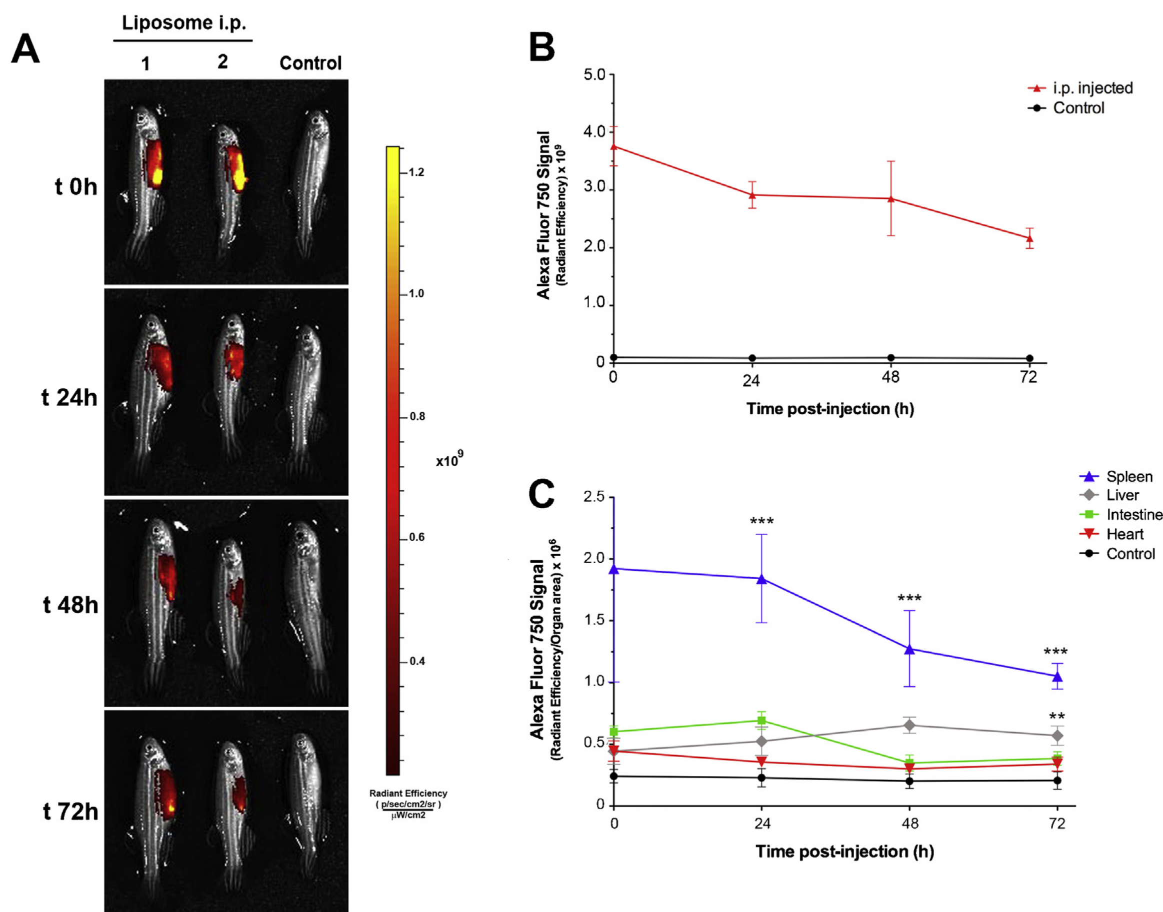

Fig. 1. Biodistribution time-course of the NL_c liposomes after i.p. injection. (A) Representative IVIS Spectrum image of adult anaesthetised zebrafish at 0, 24, 48 and 72 h after being i.p. injected with 10 μl of AF750-NL_c liposome formulation. Untreated fish were used as control for background subtraction. Image intensity is represented as Radiant Efficiency. (B) AF750-NL_c liposome fluorescent signal quantification from 0 to 72 h. Untreated fish were used as control (black dots). Image intensity is represented as mean ± SD (n = 4) Radiant Efficiency. (C) Organs including spleen, liver, intestine and heart were removed at 0–72 h and imaged to reveal accumulation of the AF750-NL_c liposomes. The fluorescence intensity of the different organs was measured using the Caliper Living Image software. Data represent the mean ± SD (n = 4) Radiant Efficiency/organ area. Differences were analysed using One-way ANOVA followed by Tukey's post-test. $^{**}p < 0.01$; $^{***}p < 0.001$.

(Fig. 2C). The membrane-staining and the z-stack images enabled visualisation of the exact location of the liposomes, and the images demonstrated that the liposomes had been completely taken up by the cells; no fluorescent NL_c liposomes attached to the plasma membrane were detected (Fig. 2B and C(iii, iv)).

3.3. NL_c injected liposomes protect zebrafish against *P. aeruginosa* lethal challenge

In previous work, we showed that NL_c liposomes induced the expression of immunologically relevant genes *in vitro* [18]. Having determined, in the present work, that these liposomes target macrophage-like cells *in vivo*, we next studied the protective effect of the system against *P. aeruginosa* infection. Before the immunisation experiments, the PAO1 infection model in adult zebrafish was fully characterised by determining the $LD_{50} = 5.3 \times 10^7$ cfu (supplementary Fig. 1), and then recovering and subsequently identifying the PAO1 strain by 16S rRNA sequencing (data not shown). The zebrafish were immunised with the NL_c liposomes, and then challenged with the PAO1 bacteria at 1 day, 1 week or 1 month post-immunisation. Their survival rates were assessed and the results were used to compare the different immunisation protocols (Fig. 3 and supplementary Fig. 2 and Table 1). Neither the empty liposomes nor the mixture of free immunostimulants (poly(I:C) and LPS) protected the zebrafish against PAO1 infection when injected 1 day (supplementary Fig. 2) or 1 week (Fig. 3A) before the challenge. In contrast, the fish that had received NL_c liposomes exhibited significantly higher survival rates than the control group, regardless of the date of administration (RPS of 33.2% at 1 day; 47.1% at 1 week; and 36.3% at 1 month (Fig. 3, supplementary Fig. 2 and Table 1). To determine the feasibility of using a storable version of the NL_c liposomes (supplementary Fig. 3), we also evaluated the efficacy of lyophilised NL_c liposomes against *P. aeruginosa* infection. Thus, adult zebrafish were treated with rehydrated lyophilised NL_c liposomes or with freshly prepared NL_c liposomes, and then infected at 1 week post-injection (Fig. 3A). Interestingly, the lyophilised liposomes were as effective as the freshly prepared ones (58.3% survival *vs.* 50% survival, respectively; Fig. 3A). This result confirmed that lyophilised liposomes are amenable to use after long-term storage.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.05.010.

3.4. NL_c liposomes administered by i.p. injection protect zebrafish against Spring Viraemia of Carp Virus (SVCV) lethal challenge

The protective efficacy of NL_c liposomes *against Spring Viraemia of Carp Virus* (SVCV) administered by i.p. injection was assessed in adult zebrafish. The fish were treated with NL_c liposomes, empty liposomes, the mixture of free immunostimulants (poly(I:C) and LPS) or PBS. At 7 days post-injection, all the fish were subjected to an immersion challenge with SVCV (Fig. 4). Similarly to the

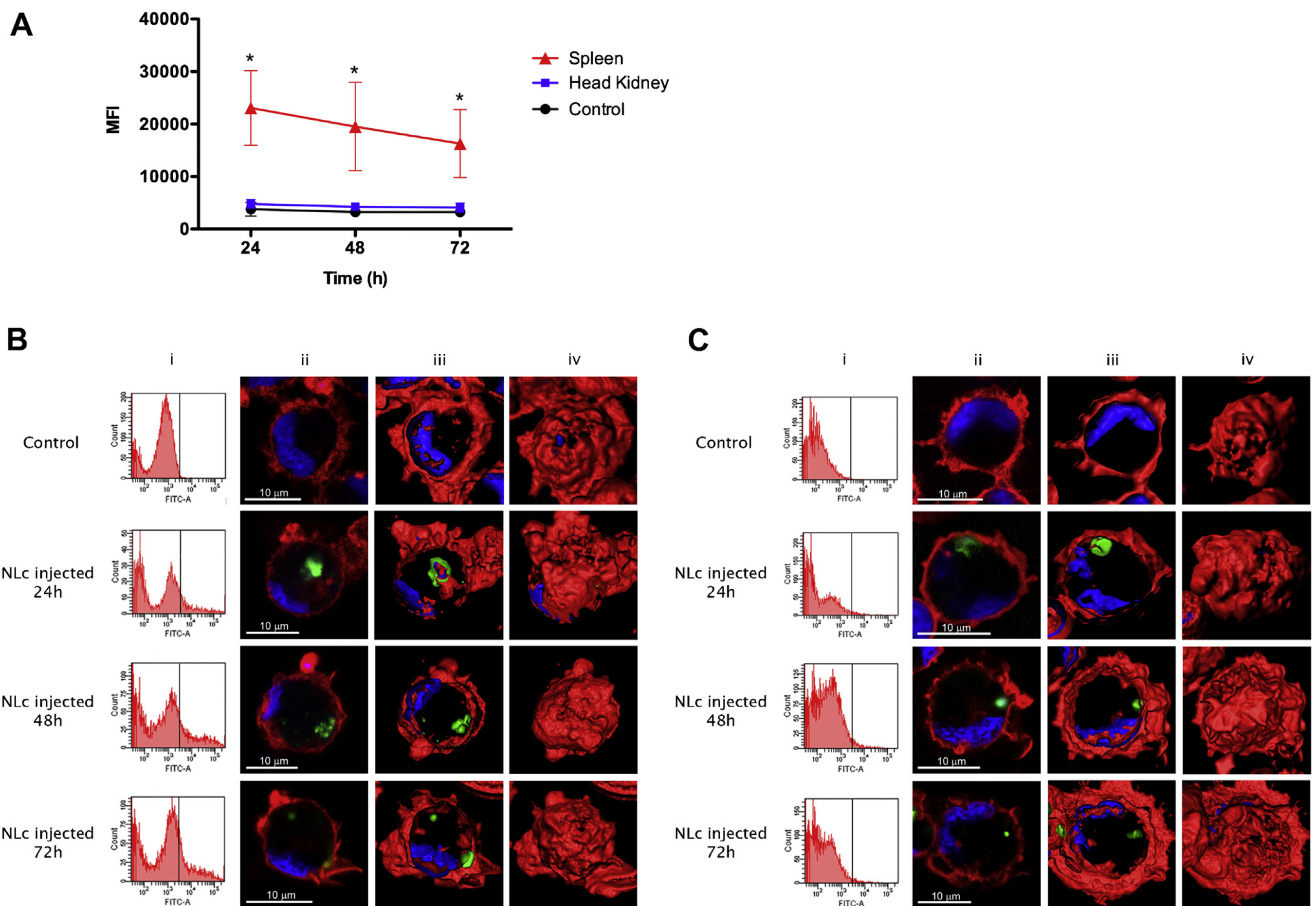

Fig. 2. Uptake of NL_c liposomes by phagocytes from trout spleen and head kidney. (A) Quantification of NL_c liposomes uptake in spleen phagocytes and head kidney phagocytes at 24, 48 and 72 h. FITC-NL_c liposomes were i.p. injected into adult rainbow trout. Cells from untreated individuals were used as control (black dots). Data represent mean ± SD ($n = 4$) fluorescence intensity (MFI) and differences were analysed using One-way ANOVA followed by Tukey's post-test. $^*p < 0.05$. Time-course of FITC-NL_c liposome uptake in spleen phagocytes (B) and in head kidney phagocytes (C). Representative flow-cytometry plot of FITC positive cells (i) and corresponding confocal images of FITC-NL_c liposomes (green) internalised in macrophage-like cells (ii). Cell membranes are shown in red and nuclei, in blue. Z-stack (iii) and whole-membrane (iv) digitalised image of the same cells.

bacterial challenge neither the empty liposomes nor the mixture of free immunostimulants offered any significant protection relative to the control fish, as measured at 15 days (RPS of empty liposomes: 0%; free immunostimulants: 7.7%). Only the fish that had received NL_c liposomes showed a significantly higher survival rate (RPS of 42.3% after 15 days) (Fig. 4 and supplementary Table 1). This difference was evident throughout the entire experiment.

3.5. *Biodistribution of NL_c liposomes in zebrafish after administration by immersion*

We also evaluated the biodistribution of fluorescently labelled NL_c liposomes (AF750-NL_c liposomes) in zebrafish following administration by immersion. The zebrafish were treated by placing them into water tanks containing AF750-NL_c liposomes. At 0 h, fluorescence was detected in the gills of all fish and by 12 h post-immersion, fluorescence was still detected in the gills but was also detected in the abdominal region of most of the fish (83.3%) (Fig. 5A). To accurately gauge the organ distribution of the NL_c liposomes, *ex vivo* imaging was performed at 12 h post-immersion (Fig. 5B). Fluorescence was observed in the gills of all fish (100%), and in the intestine and the liver of some fish (83.3% and 50% of fish, respectively). Thus, the results suggest that the NL_c liposomes had attached to the gill surface, and that they had reached the liver and the intestine. We cannot discard that NL_c liposomes also reached the intestine by the fish having swallowed water during immersion [33].

3.6. *NL_c liposomes administered by immersion protect zebrafish against Spring Viraemia of Carp Virus (SVCV) lethal challenge*

Having confirmed that these liposomes can be administered by immersion, we then evaluated their efficacy by the latter route against SVCV immersion challenge. In this case, the empty liposomes and the mixture of free immunostimulants gave a slight increase in the survival at 13 days: RPS was 20.0% with empty liposomes, 21.4% with free poly(I:C)/LPS (Fig. 6 and supplementary Table 1). However, the only statistically significant difference in the entire survival curve was observed in the NL_c liposome-treated fish, whose mortality was clearly delayed throughout the experiment (RPS value of 33.3%) (Fig. 6 and supplementary Table 1).

4. Discussion

Our experiments on NL_c liposomes administered to adult zebrafish by i.p. injection clearly indicated that the spleen was the main organ in which the liposomes had accumulated. This finding is consistent with the fact that the spleen is amongst the most important organs for filtering out foreign agents [34] and is the main organ for antigen presentation in teleost fish [31]. Furthermore,

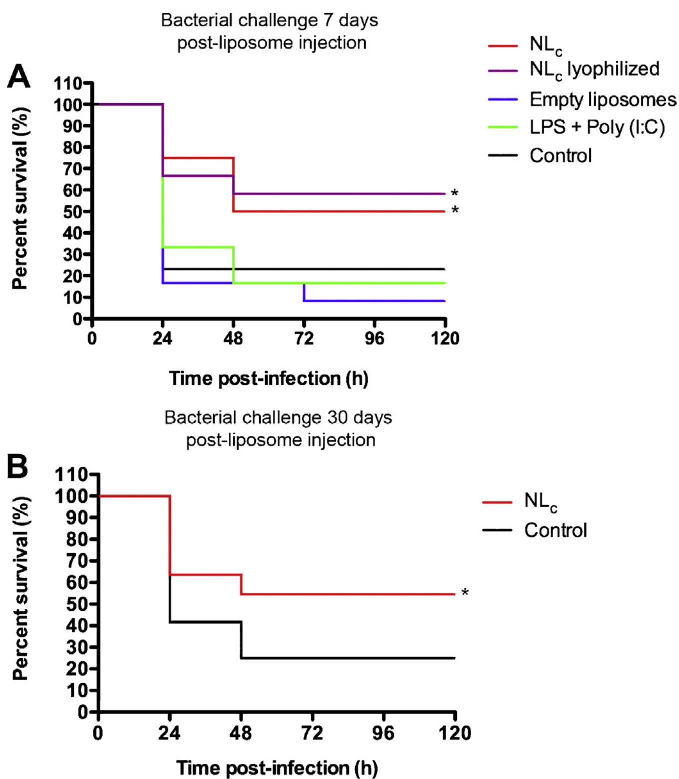

Fig. 3. Survival of adult zebrafish after i.p. injection of NL_c liposomes and challenge with *P. aeruginosa* (PAO1). (A) Fish were i.p. immunostimulated with NL_c liposomes, empty liposomes or free Poly (I:C)/LPS 7 days before being challenged with PAO1 at the LD_{50}. NL_c liposomes lyophilised for 4 months were also used. (B) Fish i.p injected with NL_c liposomes were also challenged with PAO1 one month after immunostimulation. Untreated zebrafish that had been infected with PAO1 at the LD_{50} were used as mortality control. Differences were analysed using log rank test. $^{*}p < 0.05$.

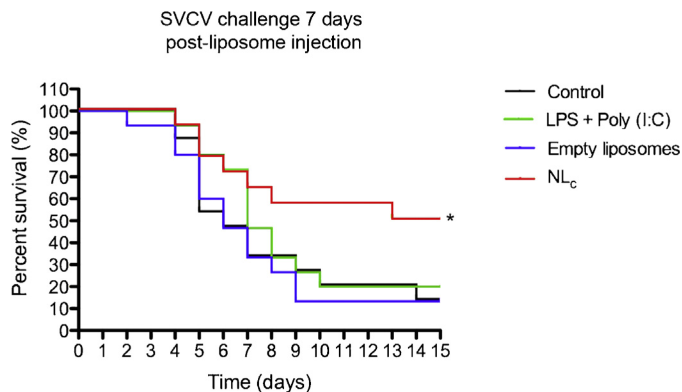

Fig. 4. Survival of adult zebrafish after i.p. injection of NL_c liposomes and immersion challenge with *Spring Viraemia of Carp Virus* (SVCV). Fish were immunised with NL_c liposomes, empty liposomes or free Poly (I:C)/LPS by i.p. injection 7 days before being challenged with SVCV by immersion. Untreated zebrafish that had been infected with SVCV were used as mortality control. Differences were analysed using log rank test. $^{*}p < 0.05$.

this result is in agreement with those of previous studies, in which the uptake and retention of injected bacteria, vaccine antigens and liposomes were demonstrated in the spleen and the head kidney [35,36]. However, we did not detect any fluorescent signal in zebrafish head kidney *in vivo*, although this was probably related to the detection limit of the method. Nevertheless, our experiments on NL_c liposomes administered to adult rainbow trout by i.p. injection demonstrated that the liposomes had accumulated in macrophage-like cells extracted from the spleen and, to a lesser extent, from the head kidney. These cells were identified as macrophages by their size, phagosome-rich cytoplasm, characteristic kidney-shaped nuclei and membrane rugosity [31,32].

The NL_c uptake mechanisms *in vivo* probably would be different depending on the tissue. *In vitro* trout macrophages internalised the NL_c liposomes mainly through caveolae-mediated endocytosis and phagocytosis, while zebrafish hepatocytes (ZFL cells) internalised the NL_c liposomes through caveolae-dependent and clathrin-mediated endocytosis [18].

The difference in the amount of NL_c liposomes found in spleen and head-kidney macrophages could be explained by the fact that the majority of the circulating monocyte/macrophages would migrate to the spleen after mobilisation to the inflammatory site [37]. Another possible explanation might be that macrophages isolated from different tissues exhibited different phagocytic responses [38]. Macrophages help regulate the immune response by producing cytokines and interferons and by presenting antigens to lymphocytes [39]. Therefore, targeting the delivery

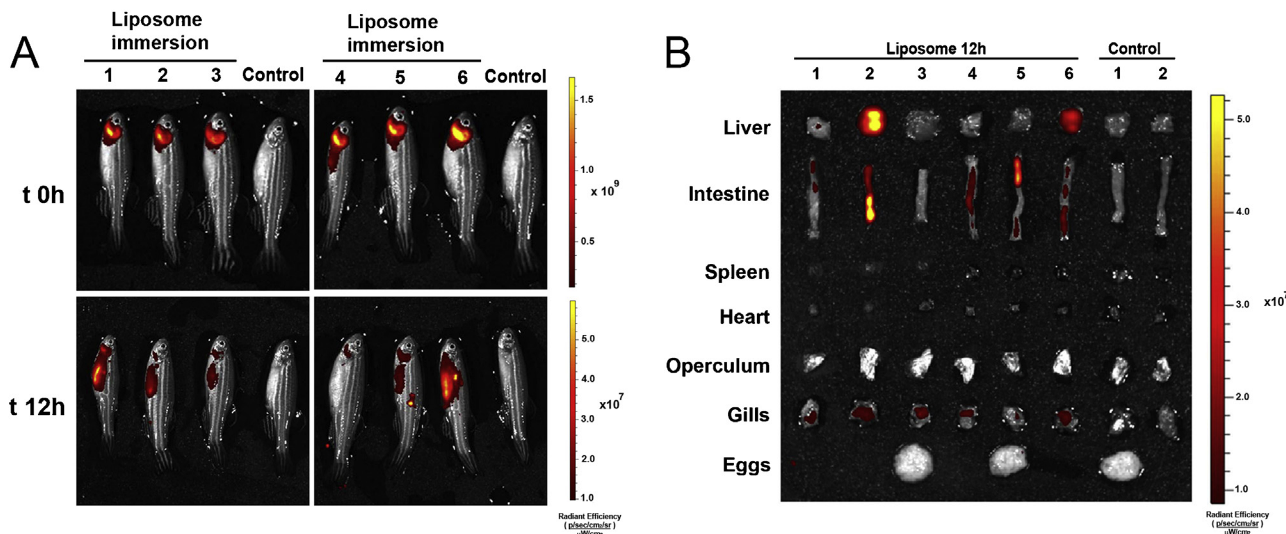

Fig. 5. Biodistribution of the NL_c liposomes after immersion. (A) IVIS Spectrum images of adult anaesthetised zebrafish ($n = 6$) at 0 and 12 h, after 30 min immersion in water containing AF750-NL_c liposomes. (B) Organs (including liver, intestine, spleen, heart, operculum and gills) and eggs were removed at 12 h and imaged to reveal accumulation of AF750-NL_c liposomes. Image intensity is represented as Radiant Efficiency. Non-immersed fish were used as control for background subtraction.

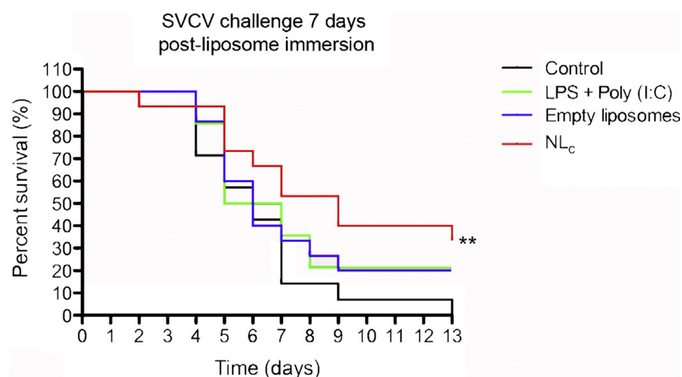

Fig. 6. Survival of adult zebrafish after bath immersion with NL_c liposomes and immersion challenge with *Spring Viraemia of Carp Virus* (SVCV). Fish were immunostimulated by immersion in water containing NL_c liposomes, empty liposomes or free poly(I:C)/LPS 7 days before challenge. Untreated zebrafish infected with SVCV were used as mortality control. Differences were analysed using log rank test. $^{**}p < 0.01$.

systems to these cells should be an excellent strategy to achieve optimal protection levels.

To test whether the NL_c liposomes could protect fish against bacterial infection, we developed a new model using *P. aeruginosa*. Despite the current lack of models in adult zebrafish, researchers have developed several models of bacterial (*e.g. Streptococcus iniae* or *Mycobacterium marinum*) or viral (*e.g.* VHSV) infection in zebrafish larvae over the past few years [40,24]. However, the maturity of larval immune systems remains poorly understood. We chose *P. aeruginosa* because it is an opportunistic pathogen in fish [22] and in humans [23], is easy to handle, and is available in multiple virulence mutants. We would like to highlight that animal models of bacterial infection such as the one we developed in this work might also prove valuable in therapeutic research for humans, especially given the fact that immunosuppressed patients (*e.g.* cystic fibrosis patients) are highly susceptible to *P. aeruginosa* infection.

The level of protection against infection by *P. aeruginosa* or by SVCV that we observed in the fish treated with NL_c liposomes, regardless of the administration route, suggests the potential utility of these liposomes as a broad-spectrum tool for immunological protection of fish. Furthermore, the fact that the mixture of free immunostimulants did not offer protection in any of the infection models underscores the importance of encapsulating in liposomes to ensure optimal activation of the immune system. Although i.p. injection remains the most widely used route to administer vaccines, it suffers some disadvantages, such as stress and side-effects at the injection site [41]. On the other hand, immersion and oral administration would be the preferable methods as they involve less handling costs and stress. However, the suitability in terms of cost-effectiveness of each vaccination method will have to be studied for each particular disease/case. In regard to this, we also evaluated the use of immersion to deliver the liposomes, as this method – in addition to being less time- and cost-dependent – offers another major advantage: the vaccine generates mucosal immunity at the site on the organism's body at which it is most likely to encounter the pathogen [42]. Thus, liposomes not only protect encapsulated actives, they also enhance the immune response by increasing mucosal adhesion [12,43].

In the present work, we found that the NL_c liposomes had accumulated in the gills, where they most likely attached to the epithelial cells and underlying phagocytes [33], and in the intestine, another reported route of antigen entry in bath-immunised fish [44,33]. The presence of NL_c liposomes in the liver following administration by immersion might be down to this organ's role in detoxification and lipid-processing [34]. This observation is consistent with previous studies in which encapsulated LPS was found in the liver after oral administration, indicating that they undergone intestinal absorption [45]. Although there have been reports of failed attempts at using immersion to administer vaccines [46], this failure might be related to the vaccine composition or because the use of the same route for vaccination and experimental challenge is probably very important [9,11]. Accordingly, we used an immersion infection model, observing a significant increase in the survival and a delay in the mortality. Thus, given the promising results we have obtained with NL_c liposomes and the fact these liposomes, once lyophilised, can be easily stored for long periods of time without losing their efficacy, we are confident that this approach will ultimately prove fruitful for use in diverse therapeutic contexts.

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