Biodistribution of surfactant-free poly(lactic-acid) nanoparticles and uptake by endothelial cells and phagocytes in zebrafish: Evidence for endothelium to macrophage transfer.

Julien Rességuier, Jean-Pierre Levraud, Nils Dal, Federico Fenaroli, Charlotte Primard, Jens Wohlmann, Gabrielle Carron, Gareth Griffiths, Dominique Le Guellec, Bernard Verrier

To cite this version:

HAL Id: hal-03281952
https://hal.archives-ouvertes.fr/hal-03281952
Submitted on 12 Jul 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
Biodistribution of surfactant-free poly(lactic-acid) nanoparticles and uptake by endothelial cells and phagocytes in zebrafish: Evidence for endothelium to macrophage transfer.

Julien Rességuière a, b, *, Jean-Pierre Levraud c, 1, Nils K. Dal b, 1, Federico Fenaroli b, Charlotte Primard d, Jens Wohlmann b, Gabrielle Carron a, Gareth W. Griffiths b, Dominique Le Guelloc a, Bernard Verrier e

a CNRS, University Lyon 1, UMR 5305, Laboratory of Tissue Biology and Therapeutic Engineering, IBCP, 7 Passage du Vercors, 69367 Lyon Cedex 07, France
b Department of Biosciences, University of Oslo, Blindernveien 31, 0371 Oslo, Norway
c Macrophages et Développement de l’Immunité, Institut Pasteur, CNRS UMR3738, 75015 Paris, France
d Adjuvatis, 7 Passage du Vercors, 69007 Lyon, France

* Corresponding author at: CNRS, University of Lyon 1 - UMR5305 / University of Oslo, Department of Biosciences, Norway.
E-mail address: julien.reseguier@ibx.uio.no (J. Rességuière).

1 Both authors equally contributed to the research article.

1. Introduction

Since their introduction in the 1960s’, a wide variety of nanoparticles (NP) have been developed, although only a few of them have been clinically approved [1]. It can be argued that a major factor contributing to this situation has been the large gap that has formed between in vitro testing and in vivo experimentation. Despite its prominence as a model, the mouse shows severe limitations for imaging NP and the cells with which they interact. Recently, the transparent zebrafish larva, which is well suited for high-resolution live-imaging, has emerged as a powerful alternative model to investigate the in vivo behavior of NP. Poly(D,L-lactic acid) (PLA) is widely accepted as a safe polymer to prepare therapeutic NP. However, to prevent aggregation, many NP require surfactants, which may have undesirable biological effects. Here, we evaluate ‘safe-by-design’, surfactant-free PLA-NP that were injected intravenously into zebrafish larvae. Interaction of fluorescent NPs with different cell types labelled in reporter animals could be followed in real-time at high resolution; furthermore, by encapsulating colloidal gold into the matrix of PLA-NP we could follow their fate in more detail by electron microscopy, from uptake to degradation. The rapid clearance of fluorescent PLA-NP from the circulation coincided with internalization by endothelial cells lining the whole vasculature and macrophages. After 30 min, when no NP remained in circulation, we observed that macrophages continued to internalize significant amounts of NP. More detailed video-imaging revealed a new mechanism of NP transfer where NP are transmitted along with parts of the cytoplasm from endothelial cells to macrophages.
preparing safe-by-design PLA-NP without surfactants and with levels of residual solvent that are in agreement with the European pharmacopoeia for human use [7,8]; this idea also fits well with general recommendations to simplify the procedures for making NP [9,10]. Both vaccine [11] and mRNA-vectorization [12] applications have been successfully made and characterized with such PLA-NP. However, like other safe-by-design or surfactant-free NP system, their biological interactions and fate after administration remain poorly understood.

The zebrafish larva is a vertebrate model with the great advantage of being highly transparent, thereby allowing researchers to analyze the biodistribution of NPs at high resolution in real time and in a non-invasive manner [13,14]. Moreover, genetically modified fish are available that selectively express different colored fluorescent proteins in specific cell types. Altogether, the zebrafish model emerges as a reliable intermediary model to bridge in vitro experimentation and in vivo experimentation in higher vertebrate. In a previous study, we have exploited this powerful system to analyze the uptake of PLA-NP by mucosal surfaces [15]. In this work, we monitored the uptake and fate of safe-by-design, surfactant-free PLA-NPs after intravenous injection. PLA-NPs were cleared from the blood in less than 30 min, and took several days to disassemble. We identify endothelial cells and macrophages as the two cell populations that internalize PLA-NPs, and describe the unexpected transfer of PLA-NPs, along with some cytoplasmic material, from vascular endothelial cells to macrophages.

2. Results

2.1. Characterization of safe-by-design fluorescent PLA-NP

Following a safe-by-design strategy to produce nanocarriers as simply as possible, but having high biocompatibility, Poly(D.L lactic acid) particles, were prepared by nanoprecipitation without the addition of surfactant or stabilizer. During this process, the small length of the PLA (<50,000 g/mol) chains enables enough negatively charged carboxyl groups to be exposed at the surface of the PLA-NP, ensuring colloidal stabilization [16]. Importantly, residual solvents (water and acetone) were gently evaporated until their minimal concentration respected the standards established by the European pharmacopoeia for human use, as assessed by gas chromatography quantification (data not shown). The visualization of PLA-NP formulations was made possible by the encapsulation of different hydrophobic fluorophores into the polymeric matrix. As measured by dynamic light scattering (DLS), we obtained highly homogeneous (Polydispersity index < 0.05) suspensions of red, green and far-red fluorescent PLA-NP which displayed Z-average of 174 nm ± 7 nm (mean(SD)), and strong anionic zeta potentials around –55 mV (Table 1). Throughout our experiments, we injected 3–5 nL of PLA-NP at 5 mg/mL in 3 day post-fertilization (dpf) zebrafish larvae. Since a larva weighs ~0.4 mg, this translates to approximatively 50 mg/kg. At this high dose, neither development nor health of injected larvae were visibly impaired by the injection of our fluorescent PLA-NP, reflecting their great biocompatibility.

2.2. Internalization of PLA-NP by endothelial cells from the whole vasculature

Despite their promising potential for nanomedicine, very few studies have been conducted to characterize the fate of surfactant-free NP in vivo. Following standard protocols [15,17], fluorescent PLA-NP (5 mg/mL) were intravenously injected into 3 dpf zebrafish, through either the post-cardinal vein or the inferior segment of the caudal vein plexus (Fig. S1). Our initial investigation of the PLA-NP biodistribution was performed by live-imaging, using an epi-fluorescent stereomicroscope, on transgenic fil1a:GFP fish [18], where endothelial cells express a green fluorescent protein in their cytoplasm. Note that in this transgenic line, neural crest cells and some macrophage progenitors can also express GFP.

Two hours following intravenous injection, red fluorescent PLA-NP were observed along the whole vasculature, from the tail region to the heart, brain and ocular vessels (Fig. 1: A). Furthermore, the fluorescence from PLA-NP seemed in close association with the fluorescence of endothelial cells, suggesting possible interactions with these cells (Fig. 1: A′-A″- Yellow arrowheads). To better visualize these potential interactions, we performed high-resolution 3D live-imaging using a high-speed spinning-disk confocal microscope, which minimizes phototoxicity. We observed extensive internalization of PLA-NP by the endothelial cells. Massive clusters of fluorescence from PLA-NP were especially found in those endothelial cells forming the caudal vein plexus (Fig. 1: B′-B″). In addition, less striking uptake was seen into the endothelial cells lining the caudal artery and intersegmental vessels (Fig. 1: C).

Analysis of the optical sections revealed that PLA-NP internalized by endothelial cells were contained within large intracellular compartments devoid of GFP and not within the GFP labelled cytosol (Fig. 1: B″ - lower blue panel). In addition, the fluorescence from free-circulating PLA-NP could not be detected within the bloodstream, nor at the plasma membrane surface of endothelial cells. However, large accumulations of PLA-NP were also detected in presumed phagocytes residing in the lumen of blood vessels. Intriguingly, it appeared that some of these phagocytes internalized both PLA-NP and GFP-labelled material (Fig. 1: B′ - upper white panel). A 3D reconstruction of the acquired image is presented in (Video S1).

Experiments in zebrafish embryos have shown essentially exclusive binding and internalization of different anionic NP to scavenger-endothelial cell in restricted venous sites, namely the caudal vein plexus and the dorsal aspect of the post-cardinal vein [19–22]. This uptake process was shown to be dependent on the scavenger receptor Stab2. In contrast, cationic NP were internalized by endothelial cells throughout the vasculature, independently of Stab2. In agreement with the above studies our anionic PLA-NP were massively internalized by endothelial cells that form the caudal vein plexus. Surprisingly however, our NP were also taken up by venous and arterial endothelial cells lining the entire system. Examples of localizations in the dorsal aorta, intersegmental vessels and both dorsal and ventral aspects of the post-cardinal vein are evident in (Fig. 1: C); uptake into the dorsal

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Fluorophore / PHR*</th>
<th>Diameter</th>
<th>Pdi</th>
<th>Zeta potential</th>
<th>Fish survival at 24 h post injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-BODIPY TR</td>
<td>Bodipy TR methyl ester / 0.02 %</td>
<td>182 nm</td>
<td>0.019</td>
<td>-52.8 mV</td>
<td>100 %</td>
</tr>
<tr>
<td>PLA-BODIPY FL</td>
<td>Bodipy 500-510 GdS / 0.04 %</td>
<td>171 nm</td>
<td>0.024</td>
<td>-57.1 mV</td>
<td>100 %</td>
</tr>
<tr>
<td>PLA-DY650</td>
<td>DY650-decylamide / 0.10 %</td>
<td>170 nm</td>
<td>0.018</td>
<td>-56.9 mV</td>
<td>100 %</td>
</tr>
</tbody>
</table>

* parts per hundred of resin
Fig. 1. Internalization of PLA-NP by endothelial cells and macrophages. Representative live-acquisitions of 3 dpf zebrafish, 2 h after intravenous administration of PLA-NP, using a stereomicroscope (A-A') or a spinning-disc confocal microscope (B-D'). (A-A') Fluorescent PLA-NP (magenta) appear closely associated (yellow arrows) with the whole vasculature of fil:GFP fish (green). Injection sites are usually at the post-cardinal veins (blue circle) or within the inferior region of the caudal vein. As illustrated by the maximum intensity projection (MIP), which displays both PLA-NP and endothelial cells (B) and PLA-NP only (B'), strong accumulations of PLA-NP are already present within the endothelial cells of the caudal vein plexus (white arrowheads). PLA-NP are also taken-up by the endothelial cells of the caudal artery (cyan arrowheads), (B') As revealed by optical sections (B), among both the caudal vein plexus and the caudal artery (white and cyan arrowheads, respectively), PLA-NP within endothelial cells are stored inside GFP negative cellular compartments. This localization is even more striking when the fluorescence signal from PLA-NP is removed (blue panel). Cluster of PLA-NP could be observed among luminal phagocytes (yellow stars). Co-internalization of GFP and PLA-NP within some luminal phagocytes has also been noticed (white arrow within white panel). (C) PLA-NP internalization is not restricted to the caudal vein plexus or the dorsal aspect of the post-cardinal vein. Endothelial cells from the whole post-cardinal vein (white arrowhead), the intersegmental vessels (yellow arrowhead) and the dorsal aorta (cyan arrowhead) are PLA-NP-positive, illustrating the pan-vascular internalization of PLA-NP. (D) From a mpeg1:mCherry zebrafish, MIP illustrating the presence of 7 macrophages (3D surface - red) close to PLA-NP (cyan) within the caudal vein plexus area. Both opening of the stack with a clipping plane (D') and the optic sections (D') reveal internalizations of PLA-NP by macrophage from the lumen (white arrowheads – 1, 5, 6) and outside the lumen (yellow arrowhead - 7). (B-B') 17 μm Müller (x60 objective) from which (B') is a 1 μm thick optical section. (C) 50 μm MIP (x60 objective). (D) 12 μm MIP (x60 objective) with a 3D surface reconstruction of the macrophage fluorescence signal. (D') illustrates an opening the MIP with a clipping plane, while (D') is a 1 μm thick optic section from the stack. Annotations: B, brain; CA, caudal artery; CVP, caudal vein plexus; DA, dorsal aorta; DLAV, dorsal longitudinal anastomotic vessel; E, eye; H, heart; ISV, intersegmental vessel; L, lumen; PCV, post-cardinal vein; SIV, sub-intestinal veins; Y, yolk. Scale bars: 200 μm (A), 100 μm (A', A''), 20 μm (C) and 10 μm (B-B', D-D'). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

longitudinal anastomotic vessels and the sub-intestinal vein are shown in (Fig. S2) and into the cerebral blood vessels and liver sinusoids (Fig. S3). We also localized PLA-NP inside endothelial cells forming the endocardium of the atrium and the ventricle of the heart (not shown).

2.3. Internalization of PLA-NP by professional phagocytes

In order to identify the cells that were accumulating PLA-NP inside the bloodstream at the caudal vein plexus, we focused on the phagocytes already present in the zebrafish embryo at 3 days post-fertilization: macrophages and neutrophils. Therefore, we first injected green fluorescent PLA-NP (5 mg/mL) into mpeg1:mCherry transgenic zebrafish [23], whose macrophages express a red fluorescent cytosolic protein. Two hours later, 3D live-imaging acquisitions at the caudal vein plexus, a region where macrophages are abundant, clearly showed the internalization of PLA-NP by macrophages residing within the lumen (macrophages #1-2-3-5-6) and outside the blood vessel (#7). In addition, macrophages devoid of PLA-NP could also be observed (#4) (Figure: D-D'). A 3D reconstruction of the acquired image is presented in (Video S2). By contrast, neutrophils, labelled in mpx:GFP zebrafish [24], showed no detectable interaction with red fluorescent PLA-NP, even when the particles were injected at higher concentration (10 mg/mL) (Video S3).

2.4. Dynamic of PLA-NP biodistribution from 30 min to 24 h by light microscopy

The biodistribution of NP at a given time is crucial information to understand how the particles interact with the organism and allow one to better design the therapeutic strategy. The above observations showed that PLA-NP could enter endothelial cells and macrophages in significant amounts. We next investigated the kinetics of this uptake process by regular observations (30 min, 4 h and 24 h) following injection of fluorescent PLA-NP in 3 dpf zebrafish (either fil:GFP or mpeg1:mCherry). We then focused on the co-localization of PLA-NP with respect to selectively labelled endothelial cells and macrophages.

Although we monitored live embryos, here we favored the use of cryosections over live-imaging to analyze samples at precise time points and visualize the internal organs in more detail. The cryosections were prepared with a thickness of 30 μm and imaged in the center to avoid the risk of the artefactual displacement of NP, which from our experience is inherent to thinner cryosections (< 20 μm) [15].

The high-resolution imaging of the cryosections confirmed that, once in the circulatory system, fluorescent PLA-NP were promptly taken up by endothelial cells. After 30 min following the administration (Fig. 2: A-A’), while no fluorescence from PLA-NP could be detected inside the bloodstream, strong accumulations of PLA-NP were present inside endothelial cells. These accumulations showed a scattered distribution inside the endothelial cells. At later time-points, 4 h and 24 h post-injection, the association of PLA-NP with endothelial cells was still observed, increasingly, the accumulation of NPs formed massive clusters adjacent to the nucleus (Fig. 2: B-C), presumably in endocytic organelles [25].

Like the endothelial cells, macrophages were quick to internalize fluorescent PLA-NP, as seen 30 min following the intravenous injections (Fig. 2: D). No striking changes in the localization pattern inside macrophages could be detected at later time-points (Fig. 2: E-F). Intriguingly, although based on qualitative observations, we noticed that the fluorescence signal from the PLA-NP seemed more intense in some macrophages at the 24 h time-point. In addition, during our observation on fil:GFP fish, we could observe phagocytes with high concentrations of PLA-NP outside the bloodstream at the 24 h time-point (Fig. 2: C, C’, star).

Finally, by investigating the whole fish, we could evaluate the capacity of PLA-NP to reach certain internal organs: the brain, the kidney and the liver. In the brain, aside from the internalization of PLA-NP by the endothelial cells that form the cerebral blood vessels, no leakage to the parenchyma could be observed at any time-point (Fig. S3: A-C). At this stage, the blood-brain barrier is functional [26]. We could however observe bright accumulations of PLA-NP in a few cells outside the vascular compartment at 24 h post-injection. These cells were later identified as brain macrophages, e.g. microglia (Fig. S3: D). The kidney, where glomerular filtration is operational in 3 dpf zebrafish [27], is responsible of the excretion of small material (< 8 nm of hydrodynamic diameter) [28]. We could not detect any sign of fluorescence associated to PLA-NP inside the pronephric ducts of the kidney at any time-points. In contrast to mammals, the liver of teleosts plays little role in the reticulo-endothelial system [29]. Still, PLA-NP were internalized by endothelial cells from the sinusoidal vessel, as well as by rare hepatocytes at latest time-point (Fig. S3: E-G).

2.5. Dynamic of PLA-NP biodistribution from 10 min to 7 days by electron microscopy

One of the limitations of light microscopy is that its resolution usually does not allow the direct observation of individual NP of 200 nm or below. Therefore, we complemented our observations from light microscopy with the use of transmission electron microscopy to study the biodistribution of PLA-NP at the sub-cellular level. There, we widened the time-span of the experiments (from 10 min to 7 days) to both witness the internalization of PLA-NP and evaluate their integrity over time. Because the poly(lactic-acid) polymer is non-dense to the electrons, in order to facilitate the identification of PLA-NP on electron micrographs, 6–7 nm electron-dense hydrophobic colloidal gold particles were
encapsulated into the PLA-NP. PLA-NP(gold) then appeared as pale spheroids with internal electron dense particles under the electron-beam (Fig. S4).

Within 10 min after the injection, single non-agglomerated PLA-NP were still circulating inside the bloodstream while others were already internalized by endothelial cells (Fig. 3: A). We could witness all the steps of PLA-NP internalization from their association to the plasma membrane to their accumulation into the intracellular endo-membrane system. While the precise internalization pathways in the cells remain to be characterized, some associations of individual PLA-NP with coated invaginations of the plasma membrane of endothelial cells were highly suggestive of a clathrin-mediated internalization (Fig. 3: B). In addition, figures resembling phagocytosis were also observed (Fig. 3: C). Once internalized, the PLA-NP loaded cellular compartment appeared to fuse into larger cellular structure containing numerous particles (Fig. 3: D). In contrast to the highly dynamic behavior of PLA-NP observed 10 min following the injection, 20 min later PLA-NP were heavily concentrated inside cellular compartments of endothelial cells while none remained inside the blood vessel (Fig. 3: E). The biodistribution of PLA-NP remained largely unchanged at the 4 h and 24 h time-points (Fig. 3: F-G). These results confirm and extend the observations we made from light microscopy, and reveal that the bright fluorescence clusters we observed represent in fact a strong accretion of PLA-NP that occurred after internalization in cells.

Whether internalized by endothelial cells or by phagocytes, the integrity and the colloidal stability of PLA-NP endured the first 24 h post-injection (Fig. 3: F-G). It was only after 3 days following the injection that change in the morphology of PLA-NP could be seen. Among some endothelial cells, gold particles were seen apparently being released from the matrix of PLA-NP; a possible consequence of an early PLA-NP degradation (Fig. 3: H). This change was more pronounced 24 h later (4 days post-injection). While PLA-NP remained intact in some endothelial cells (Fig. 3: I), in others, a shrinkage of PLA-NP was observed, in addition to the release of gold particles (Fig. 3: J). One week after injection, the heterogeneity of PLA-NP disassembly inside endothelial cells was striking. In some endothelial cells, the structure of the PLA-NP was entirely lost, only a paste of amorphous PLA mixed with gold particles remained (Fig. 3: K-L). In other cells, shrunked PLA-NP persisted along with released gold particles (Fig. 3: M). Finally, in some endothelial cells only aggregates of free gold particles remained visible (Fig. 3: N). These results indicate that an augmented release of an encapsulated cargo from surfactant-free PLA-NP could start around one week after intravascular administration.

2.6. Complementary information regarding the internalization of PLA-NP by endothelial cells

To deepen our understanding of the internalization of PLA-NP by endothelial cells, we complemented our investigations with additional live-imaging experiments.

Our previous data indicated that PLA-NP clearance occurs in the first 30 min following the administration. Videos of these first 30 min following the injection revealed that most of the fluorescent PLA-NP had already been taken-up by endothelial cells from both veins and arteries in the few minutes required to prepare the samples before the acquisitions (anesthesia-mounting-setting acquisition parameters) (Fig. S5: A-D’). As illustrated with Video S4, the clearance of PLA-NP from the blood seems to occur in around 20–25 min. Indeed, few PLA-NP could be detected inside the lumen of blood vessels at the beginning of the videos while this amount decreased overtime and past the 20th minute, almost none could be observed in the blood flow (Fig. S5: E-J).

We then investigated if the internalization of our anionic PLA-NP by non-scavenger endothelial cells [19] could be dependent of the amount of administered PLA-NP. In most of our previous experiments, PLA-NP were injected at a concentration of 5 mg/mL. Here we analyzed the biodistribution (30 min) of PLA-NP injected at different concentrations, ranging from 1 mg/mL to 10 mg/mL (Fig. S6-8). For all the different concentrations, the revealed biodistribution was similar to the one we previously described, suggesting that PLA-NP internalization by non-scavenger cells is not dependent on the saturation of the scavenger endothelial cells.

Campbell et al. [19] showed that internalization of numerous anionic NP, which are restricted to the scavenger endothelial cells, is charge-dependent and could be inhibited with a pre-treatment of intravenously injected sulfate dextran (40 KDa) to saturate the scavenger receptor stab2. We then decided to verify if the internalization of our PLA-NP by scavenger and non-scavenger endothelial cells followed similar rules. As dextran sulfate 40 KDa is quite toxic for fish, we applied the refined protocol published by Verweij et al. [30] which instead use a pre-treatment of dextran sulfate (500 KDa) for reduced adverse effects. This pre-treatment, with 3 mg/mL of sulfate-dextran 500 KDa 2 h before the PLA-NP injection, strongly reduced the internalization of PLA-NP by the scavenger endothelial cells while the internalization by non-scavenger endothelial seemed unaffected (Fig. S7: A-B’, D-E). In this configuration, the presence of PLA-NP in the blood flow 30 min following their injection was striking. This experiment suggests that while the internalization of PLA-NP by the scavenger endothelial cells seemed to follow the rules described by Campbell et al. [19], the internalization of PLA-NP by non-scavenger endothelial cells seemed to involve other mechanisms that are independent of the negative-charge of the particles. To evaluate if these other mechanisms could clear the PLA-NP from the blood, we applied a stronger sulfate-dextran pre-treatment (30 mg/mL) and analyzed the biodistribution of PLA-NP 3 h after the injection. We obtained similar results, indicating that the charge-dependent internalization by scavenger endothelial cells appears to be the main driving force behind the clearance of PLA-NP from the blood (Fig. S7: C-C’). Noteworthy is that significant internalization of PLA-NP by phagocytes could still be observed (Fig. S7: F).

2.7. Continuous internalization of PLA-NP by macrophages during the first 24 h

Throughout our investigations, we came across several intriguing peculiarities regarding the internalization of PLA-NP by macrophages.
For example, it seemed that macrophages could display a more intense PLA-NP fluorescence signal at 24 h than at 30 min post-injection (Fig. 2: E-F). In addition, the accumulation of PLA-NP by brain macrophages also raised the question of how could these cells access PLA-NP (Fig. S3: A-D)? Altogether, these elements suggested that the internalization of PLA-NP by macrophages might not be restricted to the first 30 min. We therefore decided to quantify the dynamic of PLA-NP internalization by macrophages in more detail. Because of the minuscule size of the NP, we combined live imaging and flow cytometry to obtain reliable quantification of the PLA-NP uptake by macrophages.

We first performed an automated analysis of the quantity of PLA-NP fluorescence that associate with macrophages using stereomicroscope live-acquisitions and an ImageJ macro previously developed [31]. In agreement with the observations from light microscopy, the automated analysis revealed an increase (plus 56%) in the amount of PLA-NP inside macrophages between 30 min and 24 h (Fig. 4: A). We then performed a flow-cytometry experiment on mpeg1:mCherry zebrafish injected with green fluorescent PLA-NP (2.5 mg/mL) to quantify the fraction of macrophage (mCherry+ cells) involved in the uptake of PLA-NP (Fig. 4: B-F). Almost 10 min after the injection, 8% of the macrophages already contained detectable PLA-NP. The fraction of PLA-NP-containing macrophages then steadily increased until it plateaued around 17% between 4 and 24 h. It is important to note that at this stage, numerous new macrophages are continuously being generated, increasing the pool of PLA-NP negative macrophages over the course of the experiment. Not only could we confirm the continuous uptake of PLA-NP by macrophage, these results also raised an important question: how do macrophages continue to internalize PLA-NP when none remains in the bloodstream, most of them being internalized by endothelial cells?

2.8. Evidence for a transfer of PLA-NP from endothelial cells to macrophages

We thus hypothesized that macrophages might take up PLA-NP from endothelial cells, and focused our efforts on 3D live-imaging after the 30 min timepoint to witness such events. With only a few cells separating it from the exterior environment, the flat endothelial layer surrounding the yolk (Yolk circulation valley) is a favorable region to perform dynamic 3D live-imaging at a high-resolution (Fig. S1). In this region, as illustrated (Fig. S10: A), macrophages are also abundant. During our preliminary image acquisitions, we could assess the internalization of green fluorescent PLA-NP by red fluorescent macrophages, as illustrated (Fig. S10: B-F, Video SS) by a video made 70 min post-injection. At start, this video display a PLA-NP negative macrophage moving to the close proximity of a cell with PLA-NP within cellular compartments, likely an endothelial cell. Following several minutes of apparent contact, the macrophage moved away, then displaying PLA-NP-loaded cellular compartments. This initial investigation suggested the intriguing possibility of a peculiar cell-to-cell transfer of PLA-NP.

We deepened our investigations with the injection of far-red fluorescent PLA-NP in double transgenic zebrafish fl:gFP and mpeg1:mCherry to reveal any potential transfer of material between endothelial cells and macrophages. Both PLA-NP+ endothelial cells and PLA-NP+ macrophages were present in the area, 30 min post-injection (Fig. 5: A). There, we could witness striking transfers of GFP-labelled fragments of endothelial cell cytoplasm, containing far-red fluorescent PLA-NP, from endothelial cells to macrophages. An illustration of the transfer is presented (Fig. 5: B-I, Video S6) with a 3D video acquired 30 min post-injection. In this video, a PLA-NP containing macrophage in the bloodstream can be seen moving very close to PLA-NP-positive endothelial cells. Then, less than 10 min later, internalization of a surprisingly large amount of GFP+ material could be observed inside the macrophage. The optical sections isolated from the video reveal the presence of PLA-NP among the GFP+ internalized material (Fig. 5: J-K). On a 3D image acquisition made after the video, the same macrophage could be seen with a mix of small PLA-NP+ / GFP- cellular compartments, a large PLA-NP+ / GFP- cellular compartment, and a bright GFP+ spot on its plasma membrane (Fig. 5: L-L`). Interestingly, the GFP fluorescence signal seemed more faint (compare Fig. 5: J and Fig. 5: L), possibly because of the acidity of the presumed late endocytic organelle where the NP resided that might be quenching the GFP signal [25].

It is important to note that during the transfer of NP, no GFP fluorescence signal could be observed inside the cytosol of macrophages, likely ruling out the formation of direct openings connecting the cytoplasm of both cells. No cell death could be observed among the donor cells and the neighboring endothelial cells. In addition, using acridine orange to further analyze cell death (Fig. 5: M) [32,33], we found that the intravenous injection of PLA-NP only induced the same level of cell death as the mock-injection after 30 min, likely caused by the mechanical injury. The fish then fully recovered 24 h after the injection, with all cell death level returning the level measure from non-injected fish. Overall, our observations indicate that NP transfer from endothelium to macrophage involve cell-to-cell handover of large cytoplasmic bundles, the precise mechanisms of which remain to be elucidated.

2.9. Evidence of a microvesicle-like transfer from endothelial cells to macrophages

During our investigation, we also encountered the transfer of microvesicle-like structures from endothelial cells to macrophage. This phenomenon is illustrated in (Fig. 6, Video S7). The video first shows the formation of a GFP-labelled microvesicle-like structure budding out of an endothelial cell from the caudal vein plexus (Fig. 6: A-B). After a few minutes, the microvesicle-like structure was then released from the endothelial cell (Fig. 6: C) into the lumen of the blood vessel. The microvesicle-like structure was then quickly internalized by an adjacent macrophage in the bloodstream (Fig. 6: D). Interestingly, the fluorescence signal of the GFP-labelled microvesicle-like structure started to fade after being taken up by the macrophage, until it was no more detectable 13 min later (Fig. 6: E-H).

Fig. 3. Fate of PLA-NP(Gold) once internalized by endothelial cells, from 10 min to 7 days post-injection, as observed with TEM. Representitives electron micrograph of ultrathin (60 nm) epon sections from zebrafish larvae injected at 3 dpf with PLA-NP encapsulating gold particles (<10 nm), 10 min (A-D), 30 min (E), 4 h (F), 24 h (G), 3 days (H), 4 days (I-J) and 7 days (K-N) post-injection. (A-D) 10 min following intravenous injection, PLA-NP are circulating within the bloodstream (A - yellow arrowhead) and internalized by endothelial cells (A - blue arrowhead). Internalization of PLA-NP by endothelial cells involve an invagination of the plasma membrane, resembling clathrin-mediated internalization (B - yellow stars), as well as figures resembling phagocytosis (C - red stars). Numerous NP are already being condensed within cellular compartment of some endothelial cells (D - magenta arrowheads). 30 min after the injection, no more PLA-NP could be observed within the bloodstream, in contrast to the massive concentration of PLA-NP within endothelial cells (E - blue arrowhead). No sign of degradation could be observed from PLA-NP internalized by endothelial cells (A, E-G - blue arrowhead) and underlying phagocytes (G - red arrowhead) during the first 24 h. Starting from 3 days post-injection, change in the colloidal stability of internalized PLA-NP was evident in some cells, encapsulated gold-particles being released from the NP-PLA matrix (H - yellow arrows). PLA-NP degradation was seemingly more pronounced 4 days post-injection, within some endothelial cells PLA-NP could be observed with a shrunk size (1 - yellow arrows) while in others there are more gold crystals free rather than inside the PLA matrix (J - yellow arrow). Finally, a week after the injection different shade of PLA-NP degradation could be observed inside endothelial cells, cellular compartment filled with a paste of amorphous PLA containing gold crystals (K-L - yellow arrows), shrunken PLA-NP with released gold crystals (M - yellow arrows) and cellular compartment where only aggregated gold crystals remain (N - yellow arrows). Annotations: EC, endothelial cell. Scale bars: 1 µm (A,E,G), 500 nm (F,H,I,J,K,M), 200 nm (B,D,I,L) and 100 nm (N). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
A

Macrophage uptake score

30 mn  24 h

B

Macrophage-NP+/Macrophages (%)

Ctrl 24h  Ctrl 10 mn  30 mn  2h  4h  24h

B’

Control -

10 mn

NP= 1.12 %

NP= 7.97 %

NP= 1.28 %

NP= 8.48 %

NP= 10.6 %

NP= 12.7 %

NP= 13.0 %

24 h

24 h Control -

NP= 15.6 %

NP= 0.68 %

NP= 17.4 %

NP= 0.78 %

(caption on next page)
The isolated optical sections from the video highlight the attachment of a GFP-labelled microvesicle-like structure to the macrophage plasma membrane (Fig. 6: I), followed by its internalization inside a mCherry negative cellular compartment (Fig. 6: J). We could not detect any PLA-NP inside the microvesicle-like structures during our observations. However, our data still do not allow us to rule out the possibility of a PLA-NP transfer by such a mechanism. Indeed, as illustrated (Fig. 6: K-L) in a 3D acquisition, we could observe the presence of PLA-NP, inside endothelial cells, that were in very close proximity to budding endothelial microvesicle-like structures. This budding microvesicle-like structures are also embraced by macrophages, that here co-internalized PLA-NP+ and GFP+ labelled materials.

3. Discussion

The objective of the present study was to evaluate the biodistribution of intravenously injected surfactant-free PLA-NP in vivo and with a high resolution using the zebrafish model. The goal behind the design of our surfactant-free PLA-NP was to follow the ‘safe-by-design’ paradigm of developing the most compatible NP for human applications [9,10]. Almost nothing is known about surfactant-free NP in vivo, hence our aim was to initiate the analysis of surfactant-free PLA-NP in vivo and not to make a direct comparison between PLA-NP with, or devoid of surfactant. Using a combination of live-imaging, cryosections and electron microscopy (EM), our study reveals rapid internalization of NP throughout the vascular endothelium of the zebrafish embryo, as well as into macrophages. We introduce a method allowing colloidal gold particle to be encapsulated in PLA-NP, opening the door for visualizing these NP at the ultrastructural level. Finally, by using automated imaging analysis, flow-cytometry and live-imaging we describe a striking transfer of NP from endothelial cells to macrophages.

In recent years the zebrafish has emerged as a powerful animal model for the study of nanomedicine in vivo, especially for live-imaging at a high resolution, as discussed in depth by Sieger et al. [34] It has, for example, recently been used for the live-imaging study of endogenous particles in the nanosized range such as exosomes [35] and tumor extracellular vesicles [36]. In our study, the negatively-charged PLA-NP were quickly internalized by macrophages and by both venous and arterial endothelial cells distributed along the entire vasculature (Fig. 1-2). The internalization of PLA-NP by endothelial cells was stronger in veins than arteries. Once internalized by endothelial cells, PLA-NP did not reach the cytosol and remained in vesicles from the endomembrane system, an aspect of their biodistribution we later confirmed using electron microscopy (Figs. 1–3).

The internalization of our PLA-NP into endothelial cells from all the blood vessels, veins and arteries, was surprising for an anionic NP. Others have described the uptake of different NP by endothelial cells. In a recent study, Campbell et al [19] demonstrated that the internalization of a wide range of negatively charged NP (liposomes, polymersomes, polystyrene NP, virus-like particles) was restricted to specific venous regions of zebrafish embryos, namely: the caudal vein plexus, the dorsal aspect of the post-cardinal vein and the primary head sinus. In these blood vessels, the highly endocytic scavenger endothelial cells resemble the mammalian sinusoidal endothelial cells. This pattern of biodistribution, where no NP are internalized by endothelial cells from arteries, has been observed in multiple studies using different families of negatively charged NP [20,21,37]. Even the recent studies on nano-sized endogenous exosomes and tumor extracellular vesicles also described this selective uptake by the scavenger endothelial cells forming the caudal vein plexus [35,36]. Hence, besides showing that our PLA NP can also be internalized by endothelial cells, our study also reveals a pattern of endothelial cell uptakes that was unprecedented for negatively-charged NP. Intriguingly, in the Campbell study [19] it was the positively charged liposomes that displayed a biodistribution similar to our PLA-NP. Our results then further highlight the difficulty of predicting NP biodistribution and raise an important question: why would our anionic PLA-NP behave like cationic NP and not like the other negatively charged NP? Interestingly, our complimentary experiment provided some insight to this question (Fig. S5-S9). The main mechanism responsible for the clearance of PLA-NP is a negative-charge-dependent internalization by the scavenger endothelial cells, in agreement with the model proposed by Campbell et al. [19]. However, they also revealed the involvement of one or several additional mechanism(s) that work in a negative-charge independent manner and that are responsible for the internalization of PLA-NP by the non-scavenger endothelial cells, either venous or arterial. In addition, these unidentified mechanism(s) do not result from a saturation of charge-dependent-mechanism involving the scavenger endothelial cells but occurs at the same time. Still, why only our PLA-NP are internalized this way and not the other described anionic NP remains a mystery.

Using thin cryosections to evaluate the capacity of PLA-NP to escape the vasculature, our results indicate that relatively few PLA-NP accumulate inside internal organs (Brain, Kidney, Liver, etc.) (Fig. 2, Fig. S3). This intrinsic targeting of endothelial cells from the whole vasculature by PLA-NP, with minimum accumulation inside internal organs, could be an interesting finding for developing strategies for the treatment of vascular-related diseases.

An important aspect of our study was to evaluate the fate of PLA-NP by both light microscopy and electron microscopy (Figs. 2-3). Although EM can visualize NP in detail it is rarely used to study the fate of nano-sized particles in vivo [13,22,35,36]. One of the challenges of using EM to study NP in vivo is the preservation and visualization of their structure in order to unequivocally identify them in electron micrographs. A novel approach we developed allowed encapsulation of electron-dense gold particles in the matrix of PLA-NP to guarantee their identification in electron micrographs. This enabled us to observe internalization of our PLA-NP by endothelial cells by clathrin-mediated endocytosis and a process resembling phagocytosis, as soon as 10 min post injection. The high activity of endothelial cells toward PLA-NP is consistent with these cells being highly active in clathrin-mediated endocytosis [38,39] and being able to phagocytose bacteria [40]. Subsequent to their internalization by endothelial cells, PLA-NP were concentrated within vesicles, likely from the endocytic system [25]. Our study reveals that while the distribution of PLA-NP remained almost...
unchanged during the first 24 h post-injection, they started to progressively lose their colloidal stability 72 h after being taken up by endothelial cells. The complete disassembly and release of gold particles from within some PLA-NP became evident after 1 week. Very few studies have been able to analyze the disassembly of NP in vivo using imaging techniques [37].

Macrophages and neutrophils are immune cells that are involved in the clearance of foreign materials. Devoid of stealth properties, such as the one conferred by polyethylene (PEG) [41] or polysarcosine [42, 43], our PLA-NP remained relatively briefly in the circulation and were rapidly engulfed by macrophages, as we expected. Many NP lacking stealth properties, such as PLGA [14], liposomes [19, 44] and polymersomes [45], have been shown to be rapidly taken up by macrophages when intravenously injected into zebrafish larvae, a phenomenon that is also seen in higher vertebrate models [44]. However, in our system there was no detectable uptake of PLA-NP by neutrophils. This was unexpected since neutrophils often internalize circulating NP [46], but it is not unprecedented [22, 45]. Neutrophils have been shown to phagocytose bacteria much more efficiently when presented on surfaces (e.g. after subcutaneous injection) than in the fluid phase (e.g. after intravenous injections) [47]. Yet, we could also not induce neutrophils to ingest PLA-NPs after subcutaneous injection (Data not shown).

During our investigation on the interactions of PLA-NP with macrophages, we could assess, using two different techniques (Flow cytometry and quantitative image analysis), a significant increase of PLA-NP internalization by macrophages from 30 min to 24 h following the injection. From this, we realized that macrophages continued to internalize PLA-NP at a time when no PLA-NP-loaded cellular structures along with GFP-labelled cytoplasmic material from endothelial cells to macrophages usually end up being internalized with an endothelial cell for several minutes, followed by internalization of endothelial GFP-labelled cytoplasmic material containing PLA-NP loaded endocytic organelles. This was followed by the macrophage separating from the endothelial cell, which appeared unharmed. After the internalization, the fluorescence (GFP) from the endothelial material did not co-localize with the fluorescence (mCherry) from the macrophage cytosol, consistent with a phagocytic process internalizing the fragments of the endothelial cell (contains both GFP and NP-containing organelles) into a compartment of the endothelial pathway of the macrophage. This process involving NP transfer from endothelial cells to macrophages appears to be distinct from multiple known mechanisms involved in the transfer of material from one cell to another. As the transfer of NP we report involves the transfer of cytoplasmic fragments, we do not consider transcytosis as a plausible candidate.

Tunneling nanotubes involve the formation of cytoplasmic bridges that connect two cells and can be either long and narrow or short and thick, the latter being able to transfer organelles such as endosomes and mitochondria [54–56]. However, it is unlikely that tunneling nanotubes are related to our observations as they involve a direct connection and mixing of the cytoplasm of two adjacent cells.

We also considered the possibility that the NP transfer we observe might be related to apoptotic events. Apoptosis is a process of programmed cell death by which remnants of the dying cell can be internalized by macrophages through the uptake of apoptotic bodies, or via a process called effecrocytosis [57, 58]. Apoptotic bodies are large extracellular vesicles (1–5 μm) that contain an enclosed fraction of the apoptotic cells they originate from [59, 60]. However, no apoptotic endothelial cells were observed in the region from which our videos were acquired and the donor endothelial cells appeared alive after the transfer. This is also consistent with the fact that at the early developmental stages we used, the vascular system of the zebrafish embryo is expanding, not regressing. Furthermore, we showed that the injection of PLA-NP triggers a transient and low induction of cell death that does not surpass the mechanical injury from a mock injection (Fig. 5). Thus, events related to the death of endothelial cell could hardly explain the increase of PLA-NP internalizations by macrophages that we monitored in the 24 h period after PLA-NP left the blood compartment.

We also envisaged the involvement of another described transfer mechanism, which shares similarities with the pattern we observed, especially the prolonged contact between the cells and the storage of the transferred cellular fragments apart from the cytosol. Trogocytosis is an event of partial phagocytosis that has been recently identified. Initially restricted to the nibbling of plasma membrane by T cells [61], since then, the definition of trogocytosis has being widened to include the partial phagocytosis of a donor cell, that remains alive in a healthy condition [62]. It has been reported that macrophages can perform trogocytosis [63], and in contrast to T lymphocytes, trogocytosed cellular fragments by macrophages usually end up being internalized.
In this study, we took advantage of the zebrafish model to deepen our understanding of the biological interaction and fate of safe-by-design surfactant-free PLA-NP after intravenous administration. Using live-imaging, flow-cytometry, light-microscopy and electron microscopy, we could follow in detail the interplays between PLA-NP, the endothelium and macrophages at the tissue, cellular and ultrastructural level. Our findings provide new insights into NP interaction with the vascular endothelium. The negatively-charged, surfactant-free PLA-NP were efficiently internalized by endothelial cells and macrophages. In contrast to other studies using anionic NP, our PLA-NP were distributed along the veins and arteries of the whole vasculature. A novelty of our approach was the encapsulation of hydrophobic gold particles into the matrix of PLA-NP to study their fate by electron microscopy, enabling analysis of their internalization by endothelial cells to their ongoing disassembly a week later.

In addition, our study is one of the first to evaluate the biodistribution of a surfactant-free NP system with a high quality of imaging and we believe that it will improve the understanding of how other surfactant-free NP can potentially behave in vivo.

Finally, a novel finding of our study is the existence of a new biological mechanism that allow cell-cell transfer of NP, from endothelial cells to macrophages. This could potentially be significant for the development of new NP therapy for vascular related disease and the targeting of macrophages. The precise mechanism and biological significance of this transfer of NP remain to be elucidated.

5. Material and methods

NP Preparation and Characterization.
Poly(lactic acid) nanoparticles were elaborated by nanoprecipitation as previously described [8,67,68] and were provided by Adjuvatis (i-particles®). Fluorophores were encapsulated using different fluorophore:PLA ratio: CellTrace BODIPY TR Methyl Ester™ (0,02% - Life Technologies), BODIPY 500-510CA,CS™ (0,04% - Life Technologies) and

Fig. 6. Microvesicle-like transfer from endothelial cells to macrophages. Representative live-acquisitions of 3 dpf mpeg1:mCherry and fli:GFP double transgenic zebrafish, 30 min after the intravenous injection of far-red fluorescent PLA-NP and using a spinning-disk confocal microscope. The acquisitions have been made in the tail region, at the caudal vein plexus. Endothelial cells, macrophages and PLA-NP are highlighted in green, red and white, respectively. (A-B) At the start of the video (9 μm MIP), close to a macrophage residing in the bloodstream, an endothelial cell from the caudal vein plexus is forming a bright GFP+ microvesicle-like structure (cyan arrowhead). (C) Nine minutes later, the microvesicle-like structure is released from the endothelial cell (cyan arrowhead), it is then quickly internalized by the adjacent macrophage (D - blue arrowhead). Once internalized, the fluorescence of the microvesicle-like structure rapidly fades (E-G - blue arrowheads) to finally disappear 13 min after its internalization (H – grey arrowhead). Optical sections (1 μm) from (C) and (D), (I) highlights the contact between the GFP+ microvesicle and the plasma membrane of the macrophage (cyan arrowhead), while (J) emphasize its internalization inside a mCherry negative cellular compartment (blue arrowhead). (K-K') A luminal macrophage, positive for PLA-NP (white arrowhead) and with a GFP+ cellular compartment (blue arrowhead) is wrapping around a budding microvesicle-like structure of an endothelial cell from the caudal vein plexus (cyan arrowhead). Fluorescence are represented as 3D reconstructions. (L-L') Orthogonal views from (K) reveal the presence of PLA-NP in close proximity to the budding-microvesicle-like structure (yellow arrowhead). Annotations: CA, caudal artery; CVP, caudal vein plexus and Lu, lumen. Scale bars: 10 um (A-J), 5 μm (K-K') and 4 μm (L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
DY650-decylamide (0.10% - DynoMic GmbH). The characterization, by
dynamic light scattering, of the size, as well as the size homogeneity of
the different PLA-NP formulations were performed using a Zetasizer
nanos apparatus (Malvern Instrument, UK).

For the electron microscopy analysis, 6–7 nm hydrophobic colloidal
gold particles (PlasmaChem) were encapsulated into the PLA-NP. For
this, 20 mg of gold particles were dissolved in dichloroethane and
poured in an acetone solution containing 90 mg of PLA before initiating
the nanoprecipitation process. Solution were then kept for 18 h of
storage in static mode to remove the pellet of any aggregated gold
particles that had not been encapsulated. After characterization, the
obtained PLA-NP(gold) displayed a mean diameter of 211,3 nm, a
polydispersity index of 0,199 and an encapsulation yield of gold parti-
cles of 90%. As verified with electron microscopy (Fig. S4), essentially
no free gold particles remained at the end of this process.

5.1. Animal care and ethic statement

Experiment were performed in accordance with the animal care
guidelines, ethical standards and legislation of the European Union,
France and Norway. Zebrafish embryos were reared and handled
using wild-type AB/Tubingen zebrafish and genetically-modified strains
(Tg(mpeg1:GFP)y1 [18] and Tg(mp5:GFP)i14 [24]).

5.2. Intravenous injection of PLA-NP into zebrafish embryos

Injection needles were produced using borosilicate capillaries
(GC100T-10, Harvard Instruments) and a pipet puller (P-97, Sutter In-
struments). The needles were loaded with an appropriate sample before
being connected to an Eppendorf Femtojet Express pump and mounted
onto a Narishige MN-153 micromanipulator that allowed movement in
μ
struments). The needles were loaded with an appropriate sample before
being connected to an Eppendorf Femtojet Express pump and mounted
onto a Narishige MN-153 micromanipulator that allowed movement in
the x, y and z planes. Zebrafish embryos (3 dpf) were anesthetized using a
solution of buffered tricaine (120 μg/mL) and 5 mL of PLA-NP (in PBS
1 × ) were injected into either the caudal vein plexus or the post-cardinal
vein using a femtojet (Eppendorf) and a stereomicroscope (Leica
DFC365FX). Unless specified, PLA-NP were concentrated at 0,5% (5 mg/
ml), such injection represent a quantity of 1.10⁶ to 5.10⁶ nanoparticles.

To perform the experiment saturating scavenger endothelial cells
with an anionic competitor, following the recommendations of Dr.
Frederik Verweij [30], fish were administered intravenously 1 mL
of dextran sulfate 500 KDa (Sigma), at either 3 mg/mL or 30 mg/mL, 2 h
before the administration of PLA-NP. Only fish with regular blood flow
were used for live-imaging at high-resolution.

5.3. Live-imaging

Following PLA-NP (0,5%) administration, stereomicroscope (Leica
DFC365FX – 1.0 × planapo lens) acquisitions were performed on 3 dpf
zebrafish larvae that were maintained under anesthesia (buffered tric-
aine: 120 μg/mL) and placed onto an agarose support.

For the high-resolution live-imaging acquisitions, zebrafish larvae
were maintained under anesthesia (buffered tricaine: 120 μg/mL),
embedded in 1,5% low-melting agarose onto the coverslip of a round
petri dish. Images/videos were then acquired at 28 °C using the Zyla
camera of a Dragonfly spinning disk confocal microscope (Andor), 40
μm pinholes and a 60×/1.2-water immersion objective. Acquisitions
were performed using the Fusion software, while image/video analysis
was made using both IMARIS and ImageJ softwares.

5.4. Toxicity and cell death assays

5.4.1. Toxicity

Zebrafish larvae (3 dpf) were injected as described above with 5 mL of
either green, red or far-red fluorescent PLA-NP (0,5%), 20 zebrafish
larvae were used per fluorescent PLA-NP. Injected fish were then indi-
vidually placed into the wells of 24 wells-plates with 2 mL of embryo
water per fish. Following 24 h at 28 °C, the number of death was
counted, taking into account lethal and sub-lethal phenotypes such as no
heart-beat, oedema, malformation and weak blood flow.

5.4.2. Cell death assay

Zebrafish larvae (3 dpf) were injected as described above with 5 mL of
either green, red or far-red fluorescent PLA-NP (0,5%), PBS 1 × or DMSO
80%. At 30 min and 24 h post-injection, fish were incubated in a solution
of acridine orange 10 μg/mL (Sigma) for 20 min. After 3 rinses in em-
bro water, zebrafish larvae were anesthetized with buffered tricaine
(120 μg/mL) and placed onto an agarose support to acquire the total
fluorescence associated to acridine orange with a stereomicroscope
(Leica DFC365FX – 1.0 × planapo lens).

5.5. Histology – light microscopy

Zebrafish larvae were injected with fluorescent PLA-NP (0,5%) as
previously described and were euthanized with an overdose of buffered
tricaine 30 min, 4 h and 24 h later. They were then immediately fixed
with a solution of methanol-free 4% PFA in hepes buffer 60 mM pH 7,4
for 24 h at room temperature, cryoprotected in a solution of sucrose 30%
for 24 h, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek
USA), flash frozen in isopentan, and sectioned using a CM1950 cryostat
(Leica). The resulting 30-μm cryosections were recovered on superfrost
plus slide (Thermofisher). Obtained sections were counterstained with
a solution of phalloidin conjugated with either Dylight-633 or Alexa-647
(3 U/mL - Life technologies), and DAPI (5 μg/mL - Sigma). Sections
were then mounted with prolong-glass mounting medium (Life technologies)
and cured for 24 h to reach a theoretical refractive index of 1,52. Ac-
quisitions were made using the Zyla camera of a Dragonfly spinning disk
confocal microscope (Andor), 40 μm pinholes and a 60×/1.4-oil im-
mersion objective. Acquisitions were performed using features from the
Fusion software, while image analysis was made using both IMARIS and
ImageJ softwares. Some acquisitions were made using a SP5 confocal
microscope (Leica).

5.6. Histology – electron microscopy

Zebrafish larvae were injected with PLA-NP(gold) as previously
described and euthanized with an overdose of buffered tricaine 10 min,
30 min, 4 h, 24 h, 72 h, 96 h and one week post-injection. They
were then immediately fixed in a solution of glutaraldehyde (1,5%) and PFA
(1%) buffered with cacodylate 0,1% pH 7,4 overnight at 4 °C. Following
rinses with a solution of cacodylate 0,1 M / sucrose 8%, samples were
incubated 1 h with osmium tetroxide. Samples were then rinsed with
distilled water, progressively dehydrated with ethanol solutions
(30-100%) and embedded in Epon at 60C degree for several days.
Following complete polymerization, 60 nm ultrathin sections were
realized and mounted onto copper grids. Finally, acquisitions without
additional contrast were made using an transmission electron micro-
scope MET PHILIPS CM120 with GATAN Orius200 2Kx2K camera with a
maximum resolution of 0,34 nm (Centre Technologique des Micro-
structures, Lyon, France). Image analysis was made using ImageJ softwares.

5.7. Quantification of PLA-NP internalization by macrophages

5.7.1. Flow cytometry

Transgenic mpeg1:GmCherry zebrafish (3 dpf) were intravenously
injected with less concentrated green fluorescent PLA-NP (0,25%) to
reduce the risk of false-positive event detection due to the presence
of non-internalized PLA-NP 10 min post-injection. Fish were euthanized by
overdose of buffered tricaine at 4 °C 10 min, 30 min, 2 h, 4 h and 24 h
post-injection. For each time-point, 10 larvae per replicate were
injected. Samples were chopped in fine pieces using microscissors and incubated in a solution of versene water (Life Technologies) complemented with trypsinine (2 g/l), under constant steering. Samples were then placed onto ice, complemented with fetal calf serum (FCS) to inhibit the digestion and processed through 40 μm cell strainers (Thermofisher). Resulting cell suspensions were centrifuged (400 g, 8 min, 4C degree) and resuspended in PBS 1× with 4% FCS. To allow the exclusion of dead cells, samples were complemented with DAPI (2.5 μg/mL) right before the acquisitions by LSRII (Bioscience) flow cytometer. Acquisitions were analyzed using the FlowJo v7.6.5 software.

5.7.2. Automated quantification

The measuring of macrophage uptake has been previously described in a recent publication [31] using a Fiji MACRO which was also used here.

For this experiment, the zebrafish strain Tg(mpeg1:mcCherry)gl23 [23] having red fluorescently labelled macrophages was used. Five nanoliters of green fluorescent PLA NP were injected in the zebrafish posterior cardinal vein and, by using a Leica stereomicroscope DFC365FX (1.0× planoapole lens), images of the whole zebrafish (30 ×) or the caudal region were taken (120 ×) at 30 min and 24 h after injection. Using the program Fiji, the fluorescence of the pixels (expressed as RawIntDen) relative to the background fluorescence of the whole zebrafish [23] was normalized by the overall fluorescence (also expressed as RawIntDen) and multiplied by 100. The formula can be expressed as follows:

Macrophase accumulation (30 min or 24 h) = \frac{\text{Raw Int Den Macrophasees (30 min, 24h)}}{\text{Raw Int Den Whole zebrafish (30 min, 24h)}} \times 100

5.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0.1. Normality of samples was tested using Agostino and Pearson omnibus normality test. For the quantification of PLA NP internalization by macrophages, populations did not display a normal distribution so a non-parametric one-tailed Mann-Whitney test was carried. For the cell death assay, all populations were displaying a normal distribution, thus we carried a one-way ANOVA coupled with Bonferroni’s multiple comparisons post hoc analysis. Significance level is indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2021.01.006.

Supplementary material

Declaration of Competing Interest

BV and CP declare financial interest in Adjuvatis. The remaining authors declare no competing financial interest.

Acknowledgement

We thank the Norwegian Research Council for funding (FRIMEDBIO-No 144642). This work was also supported by the ANR project FishRNAVax (ANR-16-CE20-0002-01 and ANR-16-CE20-0002-03) and by Euronanomed III (Flunanaor).

We thank the PRECI fish facility (L.Bernard and R.Renard), the AniRA-Cytomètre platform (T.Andrieu and S.Dussurolly), the PLATIM-Microscopy platform of SFR Biosciences Gerland-Lyon Sud (UMS3444/US8). We would also like to thank the Primatiss histology platform (C.Lehias and N. El Khiol) from IBCP, the CThrä Centre Technologie des Microstructures electron microscopy platform from UCBL, the EM-lab from the university of Oslo (N.Roos and head engineers), the fish facility (A.C.Tavara and J.Santana) of NCMM, the Oslo NorMIC imaging platform (O.Bakke, F.S.Kjeldal and L.Haugen). We address a special thanks to E.Delaune, S.Essayar, D.Ficheux, V. Bjørnestad, F.Verweij, D.Frei and B.Mathiesen for their advices and assistance.

References


