Toxicity, uptake and gene expression studies of nanoliposomes used as vaccine delivery systems in aquaculture

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Intensive aquaculture often involves high pathogenic burdens in farms that can provoke disease outbreaks accounting for immense economic losses, being the development of protective/vaccination strategies a priority research area for aquaculture industry [1]. Although there are a number of commercial finfish vaccines, the initial expectations have not been fulfilled because the achieved protection levels are usually low, particularly for viral vaccines. In this field, nanotechnology, and more specifically, the use of nanocarriers could help to increase the fish immunisation levels by improving delivery of vaccines and other bioactive agents to specific immune actors (*e.g.* the innate pathogen receptors (PRRs) located on antigen-presenting cells (APCs) [2]). Furthermore, nanocarriers can also be useful for a proper administration of the adequate doses in order to not over stimulate the immune system, thus avoiding the presence of unwanted side effects.

This work presents the development and use of small unilamellar nanoliposomes encapsulating a cocktail of immunological relevant molecules as new carriers to stimulate the fish innate immune response, protecting them against a pathogenic challenge. The selected immunological relevant molecules are the bacterial lipopolysaccharide (LPS) from E. coli and the synthetic analogue of dsRNA virus, polyinosinic:polycytidylic acid (poly (I:C)). Because of this selection, it is expected that this liposomal LPS-poly(I:C) cocktail may be used as non-specific vaccine nanocarrier in different fish species in a near future [3].

Liposomes encapsulating both immunostimulants are prepared by thin film hydratation method using the DLPC:Cholesterol:Cholesteryl:PEG lipid mixture. Through this methodology can be synthesized highly homogeneous small unilamellar vesicles (Figure 1A) with an average particle size of 125.8 nm, which entrap both LPS and poly (I:C) with loading efficiencies of 22.3 % and 99.6 %, respectively. Liposomal formulation showing a positive surface charge (+1.37 mV) is ideal for encapsulating both LPS and poly (I:C). The attractive interaction between the negative charge of immunostimulants and the positive surface charge of liposomes results in the near-perfect conditions to achieve the highest encapsulation efficiencies. The occurrence of these attractive interactions is corroborated by co-encapsulating fluorescent labeled-LPS and poly (I:C) into liposomes. Confocal microscope images of liposomes demonstrate that both LPS and poly (I:C) are incorporated into their cationic lipid bilayer.

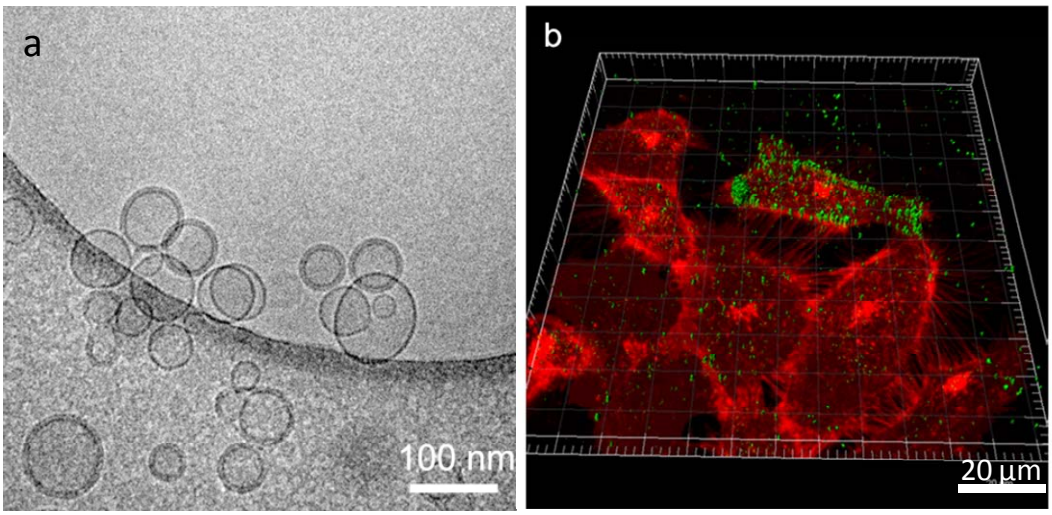

Figure 1. (a) Cryo-TEM image of DLPC:Cholesterol:Cholesteryl:PEG liposomes extruded through a 200 nm pore size membrane. (b) Confocal microscopy image of fluorescently tagged liposomes endocyted by zebrafish hepatocyte cells (3D reconstruction). Cells were incubated 30 min. with liposomes containing DHPE-Fluorescein (green).

We show that this liposomal nanocarrier presents low toxicity not only *in vitro* using three different cellular models but also *in vivo* using zebrafish embryos and larvae. Using fluorescent labeled liposomes containing both LPS and poly (I:C), it was demonstrated that such liposomal LPS-poly(I:C) cocktail is able to enter into contact with zebrafish hepatocytes (see Figure 1b) and trout macrophage plasma membranes at short incubation times, being preferentially internalized through caveolae-dependent endocytosis, although clathrin-mediated endocytosis in ZFL cells and macropinocytocis in macrophages. Importantly, we anticipate that this liposomal LPS-poly(I:C) cocktail elicits a specific pro-inflammatory and anti-viral response in both zebrafish hepatocyte cells and trout macrophages, after studying the changes in the expression of different immune related genes. The design of a unique delivery system with the ability to stimulate two potent innate immunity pathways virtually present in all fish species represents a completely new approach in fish health [4].

Further work is ongoing to evaluate the *in vivo* biodistribution and portals of entry of the liposomal LPS-poly(I:C) cocktail in three aquacultured fishes (trout, seabream and seabass), which will allow us to compare different ways of administration (injection, oral and immersion) and to design rational immunisation protocols.

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