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Molecular transfer to Atlantic salmon ovulated eggs using liposomes

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ABSTRACT

The delivery of exogenous biomolecules into teleost eggs is currently mostly relying on the manual microinjection methods, which, due to their high costs and low throughput, are not economically feasible for large-scale aquaculture applications. The main objective of this study was to develop a convenient, simple immersion delivery model for unfertilized Atlantic salmon (*Salmo salar* L.) eggs using liposomes as a system for the delivery of selected molecules.

We used a lipophilic marker, Rhodamine B isothiocyanate (RBITC) dye, to follow liposomal uptake, marker release and marker distribution within incubated egg. We investigated the influence of the incubation time, liposome surface charges and liposome sizes on the uptake into the eggs. After incubation with liposomes, the exposed eggs were fertilized and embryonic development was monitored until complete yolk sac absorption. The result showed that RBITC, was successfully delivered to the yolk of the incubated eggs. Moreover, during embryonic development, liposomal RBITC remained in the yolk sac until the yolk was completely absorbed. The findings demonstrated a novel approach for the delivery of exogenous molecules to unfertilized Atlantic salmon eggs, opening an avenue for large-scale aquaculture therapeutic applications.

1. Introduction

Aquaculture is currently among the fastest growing food-producing industries in the world (FAO, 2016). Within the aquaculture industry there is an obvious need for a large-scale delivery of bioactive molecules to eggs and developing embryos. For example, delivery of molecules that induce temporal or permanent changes in gene expression, improve embryonic nutritional status or prevent disease outbreak during the early stages of development, may all be beneficial for the industry both economically and regarding fish welfare issues. For decades, the delivery of exogenous biomolecules for manipulation of embryonic gene expression or gene transfer into fertilized teleost eggs has mostly relied on microinjections. Microinjection is a cumbrous, costly and time-consuming method and therefore not feasible for intensive aquaculture practices where thousands of eggs need to be treated at the same time (Szelei et al., 1994; Wong and Zohar, 2015). Thus, for aquaculture practices and uptake of biomolecules to large number of eggs, there is a need for more efficient, easy and reliable alternative delivery approaches.

Nanosystems are promising tools for efficient delivery of biomolecules in mammalian cell models. Among these, liposomes are the most common and extensively studied nano-carriers for targeted drug delivery (Chen et al., 2013; Sercombe et al., 2015). Key features of liposomes are their versatility, non-immunogenicity and non-toxicity (Akbarzadeh et al., 2013; Allen and Cullis, 2013; Laouini et al., 2012; Schwendener, 2014). The cargo molecules, depending on their chemical properties, can be included in different compartments of liposomes: water-soluble compounds (proteins, peptides, nucleic acids, carbohydrates, haptens) are entrapped within the aqueous inner space of liposomes, whereas lipophilic compounds (drugs, lipopeptides, adjuvants, linker molecules) are intercalated into the lipid bilayer. Such dual transport capacity opens up the possibility for efficient entrapment of a wide variety of molecules. Moreover, the versatility of such carrier systems (Sercombe et al., 2015) increases the safety and efficacy of administration (Shi et al., 2010). However, in aquaculture, use of liposome-based delivery is still in its infancy compared to mammalian application.

In aquaculture, liposomes have been, up to now, mostly used as carriers for zooplankton nutrients enrichment (Barr and Helland, 2007; Hawkyard et al., 2015) in bivalve mollusc larvae (*Venerupis decussatus* and *Venerupis pullastra*) (Lai et al., 2004) and therapeutic agents, such as immunostimulants and vaccines (Ji et al., 2015; Ruyra et al., 2013). For example, liposomes with lipopolysaccharides (LPS) from *Aeromonas salmonicida* gave rainbow trout better protection against furunculosis

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than the LPS administered alone (Nakhla et al., 1997). However, very few studies have demonstrated delivery of molecules to fish fertilized eggs or zygotes in large-scale. Such scarcity is likely due to high protection of the fertilized eggs provided by the chorion, hence, a method circumventing this limitation would be beneficial for the aquaculture industry.

In salmonids, ovulated eggs can be stored in celomic fluid or balanced salt solutions for an extended period without any significant loss of egg quality (fertilization and developmental capacity). Storage time is dependent on the temperature and media composition (Niksirat et al., 2007). In rainbow trout, this "ovulation window" was reported to be up to nine days when eggs were stored in a celomic fluid at 2–3 °C (Niksirat et al., 2007) or at least two days at 12–13 °C, when stored in a modified Cortland solutions (Goetz and Coffman, 2000). Moreover, all teleost eggs are surrounded by an extracellular protective outer envelope called zona radiata or chorion, covering the egg plasma membrane (Yamagami et al., 1992). Ultrastructure examination of the salmonid chorion shows numerous pore channels (Schmehl and Graham, 1987), which may potentially allow passage of biomolecules. After fertilization and egg activation, rapid changes in the chorion structure occur, which might limit chorion passage for larger molecules. Fertilized eggs in coho salmon have been found to take up lipophilic molecules of low molecular weight (*e.g.* sex steroids) *ex vivo* (Piferrer and Donaldson, 1994). Whether the egg chorion will allow passage of different charged molecules such as proteins or even nano-carriers, still needs to be investigated. The aim of the present work was to explore the permeability of ovulated eggs for molecular delivery. We hypothesized that utilizing the unique features of the egg during the "ovulation window" would make it the most convenient and safe period for delivering biomolecules to salmon egg for large-scale aquaculture applications.

Here, we developed a method for the *in vitro* transport of lipophilic biomolecules into unfertilized salmon eggs using incubation with liposomes of various sizes as a delivery carrier. *In vivo* bio-distribution in the fertilized eggs and developing embryos were further explored. The liposomal uptake across the chorion and through the egg plasma membrane, and distribution within the egg was monitored using Rhodamine B isothiocyanate (RBITC) as an imaging molecule. RBITC is a well-established lipophilic dye (Xiong et al., 2010). It is known to be non-toxic to immature neurons, permitting the dye-filled axons to continue their growth towards their targets within the CNS. Especially interesting for us was the fact that this dye does not leak from the labeled axons or cells (Thanos et al., 1987). Due to its lipophilicity, it is expected that it will accommodate itself within the lipid bilayers of liposomes. Furthermore, the influence of liposomal size and surface charge on the uptake efficiency was evaluated.

2. Materials and methods

2.1. Materials

Lipoid S 100 (PC, soybean lecithin, > 94% phosphatidylcholine) and egg phosphatidylglycerol sodium Lipoid EPG-Na (PG) were obtained from Lipoid (Ludwigshafen, Germany), Rhodamine B isothiocyanate (RBITC), cholesterol (Ch), sodium hydroxide (NaOH), Leibovitz (L-15) medium powder with glutamine, penicillin, streptomycin, HEPES, TES, were purchased from Life Science (Sigma–Aldrich Norway AS, Oslo). Methanol was obtained from Merck (Darmstadt, Germany). Dulbecco's phosphate-buffered saline (PBS, pH 7.4) and were obtained from Life Technologies (Paisley, Scotland). All used chemicals were of analytical grade.

2.2. Preparation of liposomes

Conventional liposomes containing RBITC were prepared by the film hydration method (Naderkhani et al., 2014). PC and cholesterol (9:1, w/w ratio; 495 mg total lipid) or PC, PG and cholesterol (9:1:1, w/w ratio; 540 mg total lipid) were dissolved without or with RBITC (10 mg) in methanol. Organic solvent was removed under vacuum using rotoevaporator system (Büchi rotavapor R-124 with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for at least 2 h at 50 mm Hg and 45 °C. The lipid films were hydrated with 10 mL of PBS (pH 7.4) to form the liposomal dispersions, namely control-PC/Ch (empty), RBITC-PC/Ch and RBITC-PC/PG/Ch. Liposomal suspensions were stored in the refrigerator (4–8 °C) overnight prior to further use.

2.3. Characterization of liposomes

Liposomes (5 mL; 49.5 mg/mL or 54.0 mg/mL, respectively) from each formulation was transferred to 10 mL beaker and placed on ice bath. The sonicator (Ultrasonic processor 500 W, Sigma–Aldrich, St. Louis, MO, USA) was set to 40% amplitude and the liposomes were exposed to ultrasonic irradiation for 1, 2 or 2 × 2 min, respectively, to obtain large and small liposomes of approximately 160 nm and 50 nm in diameter, respectively. The sonicated liposomes were stored in the refrigerator for at least 6 h prior to further use. The free RBITC (Mw 536.08) was separated from liposomally entrapped RBITC by using Nanosep 100K (MWCO 100 KDa) modified polyether sulphone ultrafiltration devices (Pall life Sc, NY, USA). The supernatant, liposomal suspension free of unentrapped RBITC, was used for further characterization. The particle size distributions of liposomes were determined by photon correlation spectroscopy (PCS) (Submicron particle sizer model 370, Nicomp, Santa Barbara, CA, USA) and zeta potential measurements were performed on a Malvern Zetasizer Nano Z (Malvern, Oxford, UK) as described according to Jøraholmen et al. (2015). The liposomes were stored at 4 °C and used in experiments within 7 days after preparation.

2.4. Morphology of liposomes: transmission electron microscopy (TEM) observation

Transmission electron microscopy (TEM) imaging was used to observe the vesicle morphology (lamellarity) of liposomes after size reduction. TEM images were recorded by using a Jeol (Tokyo, Japan) Model 1011 transmission electron microscope operating at 80 kV. To prepare the TEM samples, 5 μL liposomal suspension was dropped onto a carbon-coated copper grid directly from solution and dried prior to observation.

2.5. Ex vivo uptake of liposomal RBITC

2.5.1. In vitro culture of ovulated eggs

A modified version of the L-15 culture media with slight variation from the trout media (Nagler et al., 1994) was used for the *in vitro* culture of ovulated eggs. The modified L-15 medium was adjusted to 300 mOsm with distilled water and filtered using a 0.22-μm pore-size filter and the pH was adjusted to 7.7 using 1.0 N NaOH. This medium maintained the eggs in healthy condition for at least 5 days (own observation). The temperature for maintenance of the unfertilized salmon eggs and milt was 2–4 °C. Fresh ovulated eggs and milt of sexually mature (3–4 years) Atlantic salmon were obtained from the breeding company AquaGen, Trondheim, Norway, by an overnight transport (2 °C) to Nofima, Tromsø. Before starting the experiment, eggs were washed thrice with PBS (pH 7.4) and subsequently washed twice with modified L-15 medium. Throughout the experiments, the freshly prepared buffer and medium was maintained at 4 °C. The experiments/procedures described herein have been conducted in accordance with the laws and regulations controlling experiments/procedures for live animals in Norway, *i.e.* the Animal Welfare Act of December 20th 1974, No 73, chapter VI sections 20–22 and the Regulation on Animal Experimentation of January 15th 1996.