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# Transfection efficiency and cytotoxicity of cationic liposomes in primary cultures of rainbow trout (*Oncorhynchus mykiss*) gill cells

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**Abstract**

Immunisation of fish by immersion has been applied for inactivated, whole cell bacterins, where the gill epithelial cells are considered as one of the prime uptake sites. Antigen entry is a critical factor for delivery of vaccine antigens through the immersion route, also for DNA vaccines, and delivery systems like cationic liposomes may enhance uptake. In this study, the aim was to examine the efficiency of cationic liposomes as a means to transfect primary cultures of rainbow trout gill cells with plasmids encoding viral or reporter proteins. Furthermore, the effects of the concentration and composition of liposomes/lipoplex on the viability of the cells were evaluated. Transfection of the gill cells was possible with both plasmids following transfection with lipoplexes of a neutral charge. Low concentrations and neutral/negatively charged formulations were favourable with respect to the toxicity of the formulations. Given that the mucous barrier covering the gills is overcome, this system might be useful for the priming of the local immunity in the fish gills.


*Keywords:* DNA vaccine; Immersion; Rainbow trout gill cell

## 1. Introduction

The use of DNA vaccines in aquaculture has gained increasing interest over the recent years. The intramuscular injection is the route of delivery tested most frequently [1], the classical example being intramuscular injection of plasmids encoding the viral haemorrhagic septicaemia G protein that protects against virus challenge [2]. A major drawback is that injections can only be carried out in fish above a certain size. Immersion vaccination may be a useful alternative for the delivery of DNA to fish too small for injection. Other benefits of immersion vaccination are low labour input, less handling stress and stimulation of the immune system via the natural route of pathogen entry. Although this route of administration often is associated with more variable efficacy, immersion is an established method of vaccination in the commercial production of salmonids [3]. Recently, Fernandez-Alonso and co-workers demonstrated the potential for DNA vaccines delivered via the immersion route of delivery and protection against viral haemorrhagic septicaemia virus was obtained after immersion of rainbow trout in DNA solutions in combination with short pulses of ultrasound [4]. Expression of reporter genes in rainbow trout has also been detected following immersion in cationic liposome-formulated plasmids [5]. On the other hand, negative results have been published as well. Immersion of rainbow trout in magnetic polystyrene beads coated with a DNA-vaccine did not result in protection against infectious hematopoietic necrosis virus [6].

Presentation of antigens by dendritic cells following DNA vaccination of mammals is a potent stimulus to immune responses, particularly for induction of cell-mediated immunity and the development of cytotoxic T-cells [7]. Dendritic cells have been isolated from rainbow trout spleen [8], nonetheless, the importance of these cells in DNA vaccination of fish is not known. On the other hand, retention of antigens at the site of entry is important for induction of local immune responses

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following immersion vaccination [9,10]. The fish gill is considered as one of the uptake sites of particulate antigens and the epithelial cells are important in the initial attachment and uptake [11]. Antigen entry is probably an essential and critical factor for a successful delivery of vaccines/vaccine antigens through the immersion route. This may be a critical factor also for the delivery of DNA vaccines, and the anticipation is that a delivery system like cationic liposomes may enhance the uptake and also the expression of DNA. However, at present, it is not known how a cationic liposome delivery system interacts with rainbow trout gill cells. Moreover, the ability of such formulations to facilitate or promote transfection of rainbow trout gill cells, which may be important for induction of local immunity, remains to be investigated.

Previously, we have demonstrated that cationic liposomes interact with mucous covering the gill lamellae [12]. A mucous-free ex vivo gill system is therefore essential for an investigation of the interactions between formulations and the fish gill cells per se. Cell lines from rainbow trout gill cells are not commercially available and to increase the understanding of the cellular mechanisms involved in the multiple functions of gills, a method of growing epithelial cells from rainbow trout gills in primary culture has been developed [13].

The aim of this study was to examine the utility of cationic liposomes as a means to transfect primary cultures of rainbow trout gill cells with plasmids encoding viral or reporter genes. Furthermore, the effects of the concentration and composition of liposomes/lipoplex on the viability of rainbow trout gill cells were evaluated.

## 2. Materials and methods

### 2.1. Plasmids

The plasmid construct pcDNA3-vhsG was kindly provided by Dr. N. Lorenzen (Århus, Denmark), while pcDNA3-luc was obtained from GeneCare (Lyngby, Denmark). In both constructs, the genes encoding the viral haemorrhagic septicaemia G protein or luciferase were inserted down-stream of the immediate-early enhancer–promoter sequences of human cytomegalovirus (pcDNA3, Invitrogen, Groningen, The Netherlands).

### 2.2. Chemicals

L-α-dioleoylphosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Distearoyl phosphatidylcholine (DSPC) and sodium salt of distearoyl phosphatidylglycerol (DSPG) were kindly provided by Natterman Phospholipid (Köln, Germany). Leibowitz's L-15 medium with L-glutamine, Fungizone®, gentamicin, penicillin/streptomycin (PEST) and trypsin were obtained from GibcoBRL® (Invitrogen, Groningen, The Netherlands). Fetal Bovine Serum, Earle's Minimum Essential Medium containing 25 mM HEPES (EMEM/HEPES) and L-glutamine were purchased from BioWhittaker (Verviers, Belgium). The monoclonal antibody IP1D11 was kindly provided by Dr. N. Lorenzen (Århus, Denmark). Rabbit-anti-mouse immunoglobulins and monoclonal mouse alkaline phosphatase antialkaline phosphatase (APAAP) were obtained from DakoCytomation (Glostrup, Denmark), the colour substrate Fast Red and Mayer's Hematoxylin from Sigma-Aldrich (Oslo, Norway). The Luciferase Assay System and the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) were purchased from Promega Corporation (Madison, WI, USA) while the Bio-Rad Protein Assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were of analytical grade.

### 2.3. Preparation of liposomes and lipoplexes

The liposomes were composed of the lipids DOPE:DOTAP 1:1, DOPE:DOTAP 3:1, DSPC or DSPC:DSPG 9:1 (all ratios are weight/weight ratios). Liposomes were made according to the classical film method. Briefly, lipids were dissolved in a chloroform/methanol (2:1 v/v) mixture and the organic solvent was removed by rotary-evaporation. Glass beads were added and the films were hydrated in water above phase transition temperature of the lipids ($N_2$-atmosphere). The concentration of the phospholipid-containing liposomes was determined by the method of Rouser [14]. To form lipoplexes, cationic lipid–nucleic acid complexes [15], equal volumes of DOPE:DOTAP 1:1 and plasmid (both diluted to appropriate concentrations) were mixed, resulting in charge ratios of 0.5, 1, 2 and 5. The lipoplexes were kept at room temperature for at least 30 min before use.

### 2.4. Fish

Rainbow trout (*Oncorhynchus mykiss*) were acquired from a local hatchery and were 8–13 months old. The fish were maintained in freshwater tanks and fed ad libitum. The water was filtrated and de-chlorinated before use and the temperature in the water was 10–15 °C. To prevent the growth of fungi, the holding tanks were cleaned frequently. All fish were acclimated at the site for at least 2 weeks before the isolation of gill cells started.

### 2.5. Isolation of rainbow trout gill cells

Rainbow trout gill cells were isolated as described by Pärt et al. [13] with minor modifications. In brief, the fish were decapitated and the gill filaments excised from the arches and rinsed for $2 \times 10$ min in 10 ml PBS ($Ca^{2+}$ and $Mg^{2+}$ free) containing 200 μg/ml PEST, 400 μg/ml gentamicin and 250 μg/ml Fungizone®. The filaments were thereafter transferred to 5 ml of a trypsin solution (PBS without $Ca^{2+}$ and $Mg^{2+}$, 0.05% trypsin, 0.02% EDTA) and incubated on a rotating wheel (Test-tube LD-79 rotator, Labingo, The Netherlands) for 20 min. The cell suspension was aspirated from the tubes and filtrated through a 100 μm nylon cell strainer into a stopping solution (PBS containing 10% FBS). Remaining filaments were trypsinised for an additional 20 min and filtrated into the same stopping solution. The cell suspension was centrifuged for 10 min and the cell pellet was washed twice with 5 ml of PBS containing 2% FBS by re-suspension and centrifugation. Thereafter, the cell pellet was re-suspended in culture medium (L-15 medium supplemented with 2 mM L-glutamine, 5% FBS, 100 μg/ml PEST and 200 μg/ml gentamicin). The cells were maintained in culture flasks or well plates (Falcon®, Becton Dickinson and Co., USA) at 18 °C. After 24 h incubation, the cells were rinsed twice with PBS to remove non-attached cells and the medium was changed every second day until the experiments started.

### 2.6. EPC cells

The *Epithelioma papulosum cyprini* (EPC) cell line, originating from carp epidermal herpes virus-induced hyperplastic lesions, was first described in 1983 [16] and are now a frequently used fish cell line. The cells were obtained from European Collection of Cell Cultures (ECACC no. 93120820), and were grown in EMEM/HEPES supplemented with 10% FBS, 3.5 mM L-glutamine and 50 μg/ml gentamicin at 20–25 °C.

### 2.7. Transfection studies

#### 2.7.1. Transfection with pcDNA3-luc

The transfection studies using the pcDNA3-luc plasmid were carried out in three parallel experiments where the gill cells originated from three different fish. The cells were seeded at a concentration of 200,000 cells/well in a 96-well plate. Five days after isolation, when the cells had reached 80–100% confluency, the cells were rinsed and the complete culture medium was replaced with 75 μl

serum-free medium according to Romøren et al. [17]. 25 μl transfection mix (5 μg pcDNA3-luc/well and liposome amounts to obtain the charge ratios 0.5 (21 μg lipids/well), 1 (42 μg lipids/well), 2 (85 μg lipids/well) and 5 (212 μg lipids/well), i.e., lipoplex 0.5, 1, 2 and 5) was added to each well at day 1. The lipoplexes were of a negative (lipoplex 0.5), neutral (lipoplex 1) or positive charge (lipoplex 2 and 5). The following day, the lipoplexes and the serum-free medium were removed and the cells were rinsed with PBS before addition of the complete medium. The cells were lysed in a lysis buffer (25 mM Tris–phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton® X-100) after further 2 days. The lysates were assayed for luciferase according to the manufacturers description in a Lumat LB 9507 Luminometer (EG and G Berthold, Germany) and the total protein concentration in the samples was obtained with the BioRad assay. The results were expressed as pg luciferase per mg of total protein. EPC cells were plated at a concentration of 50,000 cells/well in 96-well plates and the transfection was performed as described for the gill cells.

#### 2.7.2. Transfection with pcDNA3-vhsG

The transfection studies using the pcDNA3-vhsG plasmid were carried out in four parallel experiments where the gill cells originated from four different fish. In these experiments, the isolated gill cells were seeded at a concentration of $2 \times 10^6$ cells/well in a 24-well plate. Five days after isolation, the cells were rinsed with PBS and 750 μl serum-free medium was added to each well together with 250 μl transfection mix (5 μg DNA per well, formulated as naked, lipoplex 1 or lipoplex 2). The following day, the lipoplexes and the serum-free medium were removed and the cells were rinsed with PBS before the complete medium was added. After further 2 days the cells were fixed in absolute methanol/acetone 1:1 (v/v), and immunohistochemical analysis was performed for the detection of G protein expression. The monoclonal antibody IP1D11 (diluted 1:100 in Tris-buffered saline (TBS)) was applied, followed by rabbit immunoglobulin to mouse IgG (diluted 1:25 in TBS), APAAP (1:50 in TBS) and the colour substrate. The cells were counterstained and photographs were taken with a Nikon Eclipse T300 inverted microscope equipped with a digital camera. EPC cells were plated at a concentration of 500,000 cells/well in 24-well plates and the transfection (lipoplex 2, 1 μg DNA per well) and immunohistochemical analysis was performed as described for the gill cells.

### 2.8. Viability assays

#### 2.8.1. Viability of cells in plates exposed to liposome/lipoplex

The gill cells were seeded in 96-well plates at a concentration of 100,000–200,000 viable cells/well. After approximately 1 week, the cells were washed with PBS before 50 μl complete medium and 50 μl liposome-, or lipoplex-formulation was added to the wells. The following day, the formulations were removed and replaced with complete medium. MTS reagent (20 μl) was added to the wells after 24 h and after another 24 h of incubation the plates were read at 490 nm. The relative viability was calculated from untreated control cells. EPC cells were plated at a concentration of 100,000 cells per well in a 96-well

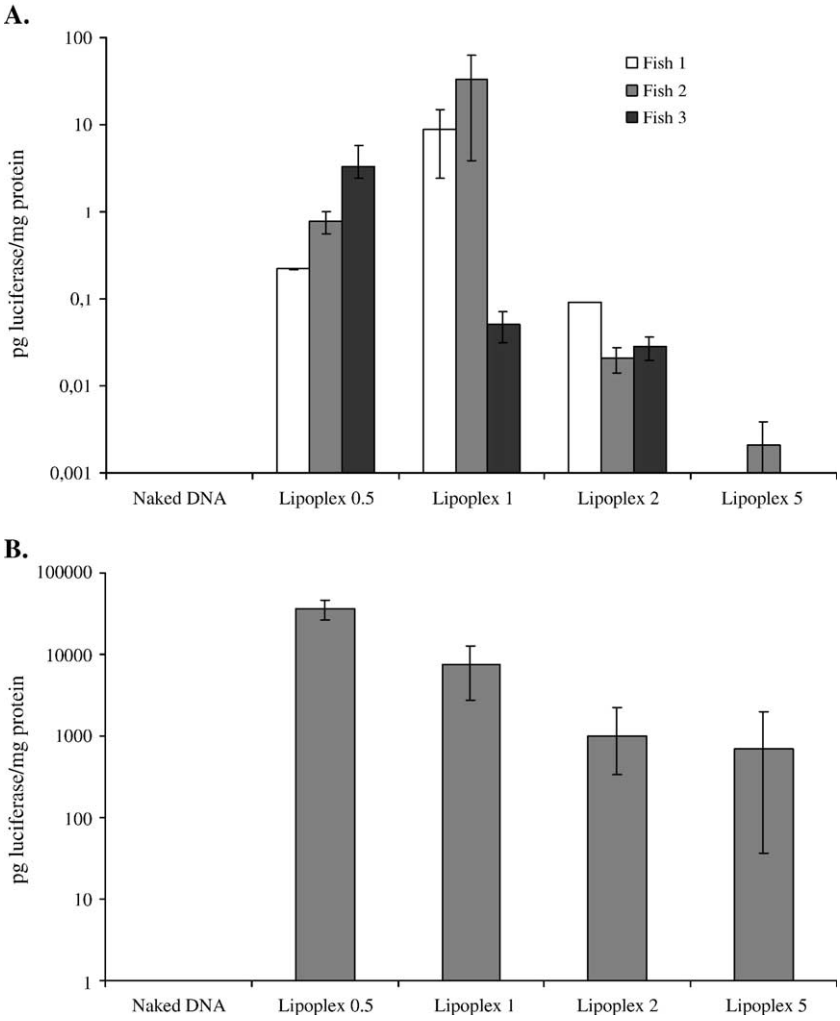


Fig. 1. The expression of luciferase (pg luciferase per mg of total protein) in primary cultures of rainbow trout gill cells (A) or in EPC cells (B). For the rainbow trout gill cells, the cells from three different fish are used and the results are given as the means of 2–3 parallels per fish ± max/min values, while the results for the EPC cells are given as the means ± max/min values ($n = 5$). 5 μg DNA is used per well in a 96-well plate. The amount of lipids used to obtain the desired charge ratios are 21, 42, 85, and 212 μg lipids/well (for lipoplex 0.5, 1, 2, and 5, respectively).

plate. After 24 h incubation, the cells were rinsed with PBS, and 50 μl formulation and 50 μl complete medium were added. The rest of the procedure was as described for the gill cells.

*2.8.2. Viability of cells grown on filters exposed to liposomes*

The rainbow trout gill cells, that in advance were grown in culture flasks for about 1 week were trypsinised, centrifuged, counted and re-seeded at a concentration of 200,000 viable cells per filter (Falcon® Cell Culture Inserts, Becton Dickinson and Co., USA). The filters had a pore size of 0.4 μm, a pore density of $1.6 \times 10^6$ pores per $cm^2$, an effective growth surface area of 0.3 $cm^2$ and were housed in Falcon 24-well cell culture companion plates. The culture medium was present both in the well (basolateral side) and the insert (apical side) and was changed every day. The transepithelial resistance, measured by a Millicell® -ERS meter connected to a pair of chopstick electrodes (Millipore Co., Bedford, MA, USA), was measured every day. The results were expressed as K ohms × $cm^2$ of cell growing surface, after subtracting the values for the resistance in the controls consisting of medium and cell culture inserts only (without cells seeded). When the TER had reached a value >1 K ohms × $cm^2$, i.e., the gill epithelium defined as a tight epithelium [18], the experiments started. 0–500 μg/ml DOPE:DOTAP 1:1 liposomes, made and diluted in synthetic fresh-water (sterile, deionised water containing 0.9 mM $Na_2HPO_4$, 0.5 mM $CaCl_2$, 0.1 mM $NaHPO_4$ and 0.05 mM $MgCl_2$) were introduced to the cells on the apical side. The transepithelial resistance was measured at predefined time-points for 2.5 h. Thereafter, the liposome formulations were aspirated, the complete medium was added on both sides of the filter and the TER was monitored for an additionally 2.5 h.

## 3. Results

### 3.1. Transfection studies

#### 3.1.1. Transfection with pcDNA3-luc

The reporter gene luciferase was expressed in the gill cell preparations from all three fish after exposure to lipoplex 0.5, lipoplex 1 and lipoplex 2 (Fig. 1A). The highest levels of luciferase for cells from fish 1 and 2 were obtained with a lipoplex 1 formulation, 9 and 33 pg luciferase per mg protein could be detected, respectively. In the cells from fish 3, lipoplex 0.5 gave the highest luciferase expression, 3.3 pg luciferase per mg protein. Lipoplex 5 gave a positive transfection in one fish only, while adding naked DNA (pcDNA3-luc in absence of a carrier) to the gill cells did not result in transfection. The highest luciferase levels in the EPC cells were obtained after transfection with lipoplex 0.5, followed by lipoplex 1, lipoplex 2 and lipoplex 5 (Fig. 1B). Noteworthy, the levels of expression in the EPC cells were at most 1000 fold higher than in the gill cells and the highest average level measured was 36 ng luciferase per mg protein (lipoplex 0.5).

#### 3.1.2. Transfection with pcDNA3-vhsG

G-protein expression was detected in gill cells from all four fish when lipoplex 1 was used for transfection. No positive identification of transfected cells was observed for lipoplex 2 or naked DNA. The transfection efficiency for lipoplex 1 was low (<0.5% positive cells) and with some variation in intensity from a faint cytoplasmic staining to a very strong expression of G protein (Fig. 2A). Pseudopodia are clearly visible in the transfected cells indicating good cell viability even after transfection. A stronger staining and hence a higher G-protein expression was detected in the EPC cells (Fig. 2B).

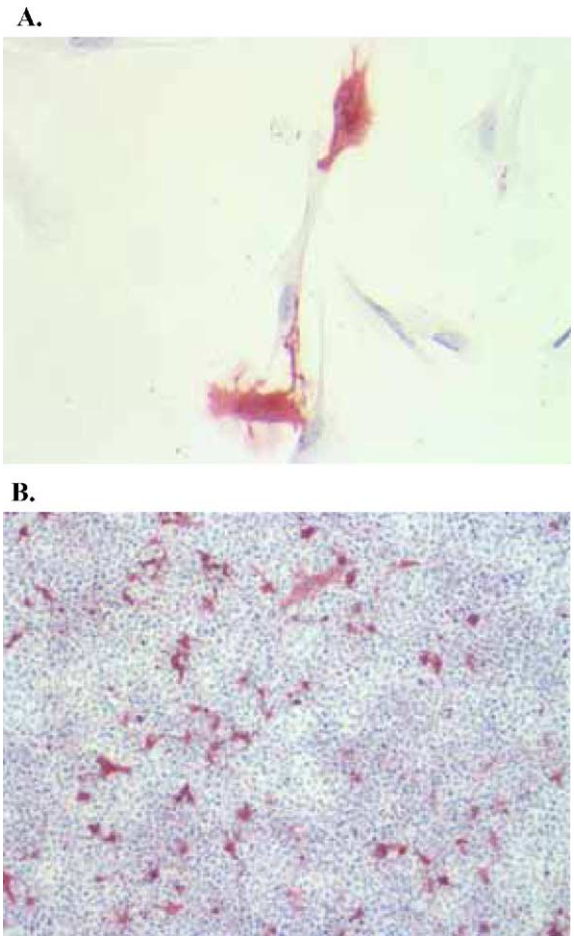


Fig. 2. Immunohistochemical detection of the VHS virus G-protein in primary cultures of rainbow trout gill cells (magnitude 40×) (A) or EPC cells (magnitude 10×) (B) transfected with pcDNA3-vhsG. The transfections were made in 24-well plates. For the gill cells, 5 μg DNA and DOPE:DOTAP 1:1-based lipoplex 1 (42 μg lipid per well) was used. 1 μg DNA and DOPE:DOTAP 1:1-based lipoplex 2 (17 μg lipid per well) was used for the EPC cells.

### 3.2. Viability assays

#### 3.2.1. Viability of cells in plates exposed to liposome/lipoplex

The relative viability of rainbow trout gill cells after exposure to different concentrations of liposomes is illustrated in Fig. 3A. Increasing the DOPE:DOTAP 1:1 concentration in the wells from 0 to 20 μg/ml decreased the viability of the cells to 42%. A further increase in the liposome concentration to 50 μg/ml reduced the viability by an additionally 29%. The viability of the cells was below 10% when the concentration was higher than 100 μg/ml DOPE:DOTAP 1:1 per well. DOPE:DOTAP 3:1, containing a smaller amount of the cationic lipid DOTAP, also exhibited high cytotoxicity. Viability was at 58% at a concentration of 20 μg/ml DOPE:DOTAP 3:1 liposomes, and at 50 μg/ml and 100 μg/ml the values were reduced to 29% and 13%, respectively. At higher concentrations, the relative viability was below 10%. The neutral and negatively charged liposome formulations, DSPC and DSPC:DSPG 9:1, did not result in such a dramatic decrease in viability. For all concentrations tested (20–1200 μg/ml) the

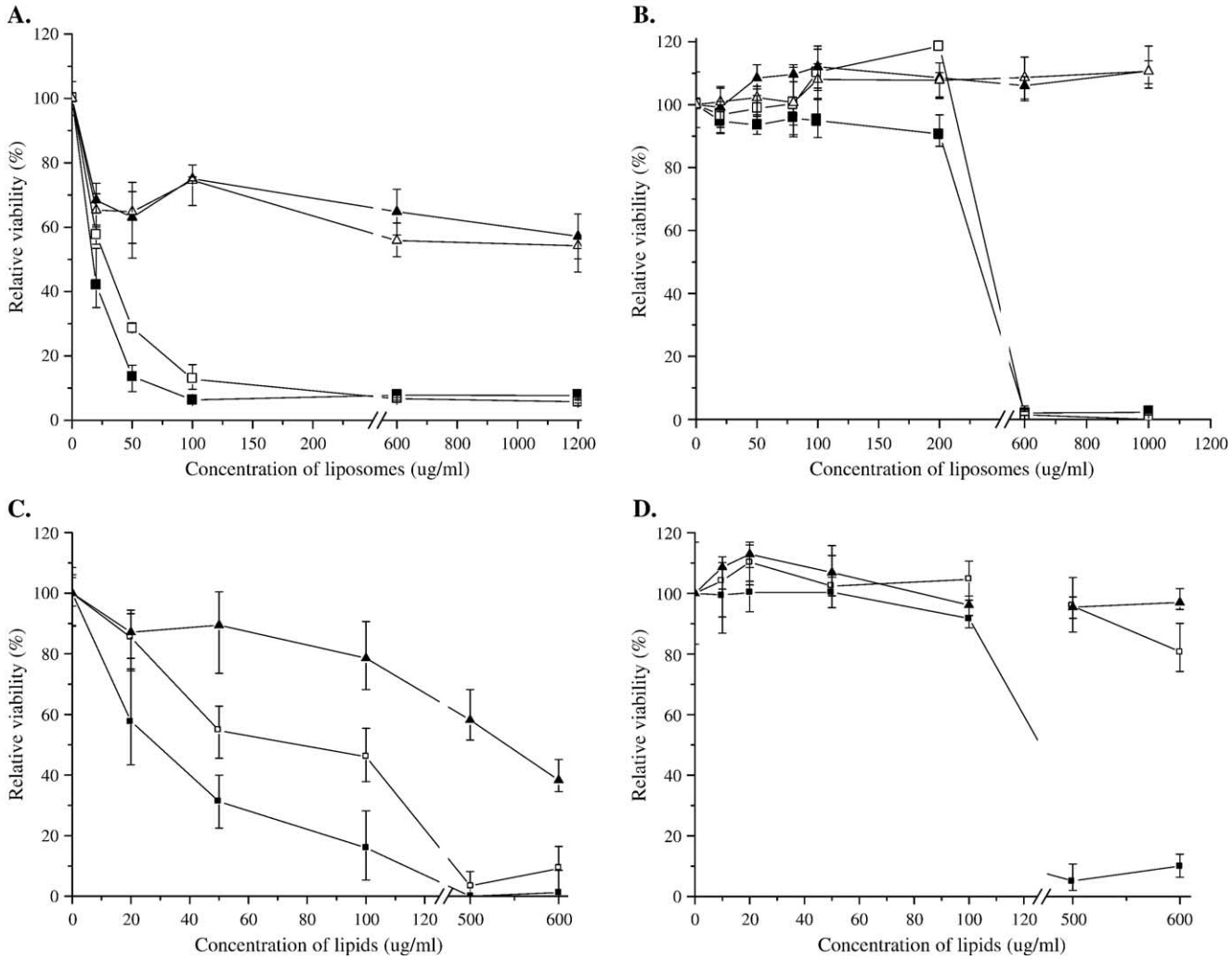


Fig. 3. The cytotoxicity of liposomes (■=DOPE:DOTAP 1:1, □=DOPE:DOTAP 3:1, ▲=DSPC, △=DSPC:DSPG 9:1) on primary cultures of rainbow trout gill cells (A) and EPC cells (B) as well as the cytotoxicity of DOPE:DOTAP 1:1-based lipoplex (■=Liposomes □=Lipoplex 2, ▲=Lipoplex 0.5) on primary cultures of rainbow trout gill cells (C) and on EPC cells (D). The values are expressed as means±max/min values ($n$=3–5).

viability was between 60 and 75%. The viability of EPC cells was not affected to the same extent when exposed to the different liposome formulations as the gill cells (Fig. 3B). In the concentration range of 0–200 μg/ml liposomes, the viability of the cells was over 90% for all liposome formulations. At concentrations of 600 and 1000 μg/ml, the DOPE:DOTAP 1:1 and DOPE:DOTAP 3:1 liposomes were found highly cytotoxic (less than 5% viable cells left). However, at these concentrations, there was no observed toxicity for DSPC and DSPC:DSPG 1:1 liposomes.

The effect of adding DNA in combination with liposomes to primary cultures of gill cells is shown in Fig. 3C, the formulations are referred to as lipoplex 2 (net positive charge) or lipoplex 0.5 (net negative charge). The toxic effect is reduced after addition of DNA to the liposomes. At a lipid concentration of 50 μg/ml, the viability increases about 2- and 3-fold when lipoplex 2 and 0.5 were used, respectively. The difference is even larger at 100 μg/ml where the viability increases around 3- and 5-fold. At a lipid concentration of 500 and 600 μg/ml, there is no difference between the viability of the cells exposed to lipoplex 2 or liposomes. 50% of the cells exposed to lipoplex 0.5, however, were still viable. The viability of the EPC cells was again higher than the gill cells and for lipoplex 2 and lipoplex 0.5, the viability of the cells was >80% for all concentrations tested (Fig. 3D).

### *3.2.2. Viability of cells grown on filters exposed to liposomes*

Transepithelial resistance is studied under asymmetrical conditions with water on the apical side and medium on the basolateral side. The fish material was limited so the experiment was only performed with DOPE:DOTAP 1:1 liposomes. As shown in Fig. 4, there was no difference in the transepithelial resistance (TER) when the gill cells were exposed to 20 or 50 μg/ml of DOPE:DOTAP 1:1 on the apical side compared to the control (synthetic fresh-water). A significant decrease in TER was observed upon raising the liposome concentration to 100 μg/ml while a liposome concentration as high as 500 μg/ml resulted in TER levels close to controls (without any cells seeded). For all formulations, the TER values decreased with time. After 150 min

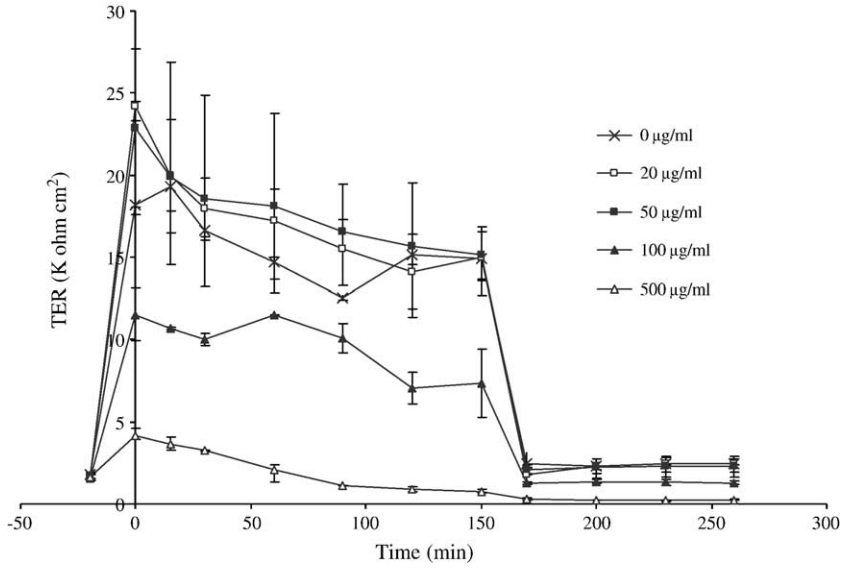


Fig. 4. Dose–response toxicity of DOPE:DOTAP 1:1 liposomes measured by transepithelial resistance (TER) of rainbow trout gill cells grown on permeable supports. The values are expressed as the means ± max/min values ($n = 2-3$).

(2.5 h), when the liposome formulations were removed and replaced with fresh medium, the cells exposed to 20 and 50 μg/ml of liposomes retained the TER prior to exposure. The cells exposed to the two highest concentrations (100 and 500 μg/ml) did not retain the TER when the liposome solution was aspirated and damage to the cells was also observed by the naked eye. Similar trends were obtained in a parallel experiment with gill cells isolated from a different fish (data not shown).

## 4. Discussion

These studies showed that rainbow trout gill cells are transfected with plasmids encoding reporter or viral proteins by use of cationic liposomes. Furthermore, the composition and concentration of the liposomes/lipoplex affected the viability of the cells and loss of cell integrity was discovered upon exposure to high concentration of cationic liposomes.

The role of the fish gills in the transfection and immune response was not examined in the DNA-immersion experiments performed by Fernandez-Alonso et al., and further studies of the mechanisms were suggested [4,5]. Induction of local immunity in the gills might be important for several reasons. In a recent study, the VHS virus was identified in the gill epithelia as soon as 1 day post-infection, indicating this being the site of entry for the virus [19]. Furthermore, retention of antigens at the site of entry is important for the local immunity to develop after immersion vaccination [9,10]. Although based on ex vivo studies, the G-protein expression observed in gill cells following transfection with pcDNA3-vhsG potentially opens up some interesting avenues to explore in more detail.

Lipoplex 1 and lipoplex 0.5 gave the highest levels of luciferase expressions in the gill cells. The highest expression levels in EPC cells were also observed following transfection with the same lipoplexes. Usually, the expression is lower when negatively charged lipoplexes are used [20,21]. In our studies, however, the amount of DNA per well was rather high, which in itself can have an inhibitory effect on transfection efficiency. Further, to obtain the correct charge ratios for the lipoplex solutions, also the lipid concentrations had to be high (up to 212 μg lipids per well in a 96-well plate, i.e., 2.12 mg/ml) and on a general basis, it is reasonable to assume that the observed toxicity for the different lipoplex formulations may cause a reduced transfection. Thus, it was necessary to carry out viability experiments.

It was clearly shown that toxicity is dependent on the type of liposomes used. The concentration of these liposomes was also of importance, and higher concentrations were more toxic than low concentrations. These findings are in agreement with studies performed on mammalian cell lines. The cytotoxicity of neutral and charged liposomes on a wide range of tumour cell lines has been reported by Mayhew et al. [22] and Cambell [23]. In these studies, inclusion of the cationic lipid stearylamine increased the cytotoxicity and it was also dependent on the amount of this lipid added per well. Also, the new generation of cationic lipids has been tested for cytotoxicity. Filion and Phillips investigated the toxicity of a wide range of cationic lipid–DOPE formulations on immune effector cells [24]. The formulations were found to be highly toxic in vitro towards macrophages and monocytes but not for T lymphocytes, which may be explained by the relative phagocytic activity of macrophages/monocytes in comparison with the T cells. In our studies, there was a remarkable difference between the two cell types and the primary cultures from the gills were more sensitive towards the liposomes than the EPC cells. A difference in phagocytic activity between EPC cells and gill cells is probably not the most likely explanation as both cells are of epithelial origin. Therefore, differences in the molecular structure and possibly charge difference on the surface of the gill cells and EPC cells may be responsible for the difference in toxicity observed for the

liposome formulations. This may be due to the origin of the fish cells (rainbow trout or carp) and the type of the cell cultures (primary cultures or permanent cell lines).

The gill epithelium consists of three cell types: Respiratory cells that constitute as much as 90–95% of the total epithelial surface area, the ion-transporting chloride cells and mucous cells [11]. When establishing primary cultures of gill cells, other cell types but the respiratory epithelial cells do not survive the isolation and culture conditions [11]. Nevertheless, traces of these may theoretically have an influence on the differences observed in transfection and toxicity in the gill and EPC cells.

The charge of the formulations will be altered by DNA inclusion in the liposomes. By an addition of DNA to cationic liposomes, the overall positive charge is reduced (lipoplex 2) or altered to a net negative charge (lipoplex 0.5), depending on the DNA concentration. Interestingly, the toxic effect of the formulations is reduced upon addition of DNA. These results are in contrast to what is described by Filion and Philips [24]. When they added DNA to cationic liposomes, a decrease in the zetapotential was observed. Nevertheless, the cytotoxicity on the macrophages used in the test was not reduced. Other studies performed with different cell lines, reported that an increased cell death was observed with increasing lipid/DNA ratios in the lipoplexes [20,25]. Such contradicting results are not easily explained, but the functions of the cells tested might provide some insight. Macrophages are professional phagocytes and will thus take up liposome particles by phagocytosis, a process involving an active process from the cell. For other cell types as in our case, the primary cultures of rainbow trout gills and the EPC cells, the interaction between the cells and the liposomes requires a close apposition where membrane charge differences become more important for uptake of the liposomes into the cells. Thus, although the mechanism by which liposomes results in loss of cell integrity and cell death might be the same, the initial adherence of the formulation to the cell surface may be of greater importance for the toxicity in cells that are less phagocytic in nature.

Epithelial gill cells held in primary culture can be grown on permeable supports and they differentiate and retain their polarised properties [11]. The differentiation and polarisation may result in a change of properties compared to primary cultures grown in plates and they are most likely more similar to the gill cells in vivo. In addition, such studies enable exposure of the cells to asymmetrical conditions, water on the apical side and medium on the basolateral side, which further resembles an in vivo situation. The viability of the cells after exposure to DOPE:DOTAP 1:1 was also determined on cells grown on filters and revealed a toxicity depending on the concentration of the liposomes. Only high concentrations of liposomes influenced the transepithelial resistance. Another interesting observation was the reestablishment of resistance after liposome-exposure. A recovery implies that the cellular integrity is maintained and that the gill epithelium still could be defined as a tight epithelium [16]. The cells exposed to the two highest concentrations (100 and 500 μg/ml), however, did not retain the TER from prior to exposure and were also visibly damaged. To our knowledge, no such studies have been performed with liposomes earlier.

The challenge with cationic delivery systems like cationic liposomes and the cationic polymer chitosan, is their acute toxicity to rainbow trout fry above a certain threshold concentration during immersion [12,26]. The mechanism probably involves an interaction between the cationic formulation and anionic components of gill mucin. It is therefore interesting that the formulations tested herein that gave the highest transfection levels and were the least toxic in vitro were the theoretically neutral and negatively charged lipoplex 1 and lipoplex 0.5, these formulations are also less likely to react with anionic components of the gill mucin. More sophisticated delivery systems, e.g., inclusion of ligands in non-toxic liposome formulations may hold a potential. Yet, such systems are most likely too expensive for fish vaccines and too complicated for mass production. There is a great challenge to overcome the mucous barrier of the gills, but given that it can be achieved, there should be potential for induction of local immunity in the fish gills using DNA vaccination.

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