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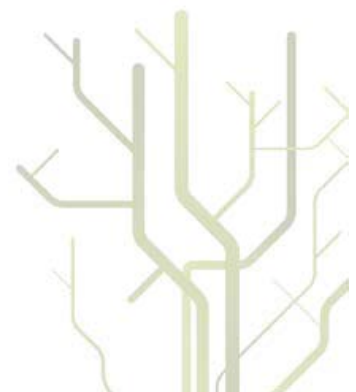
PLGA (poly(D,L-lactic-co-glycolic)acid) particles as DNA carriers in Atlantic salmon (*Salmo salar* L.)

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A dissertation for the degree of Philosophiae Doctor
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**There are no facts,
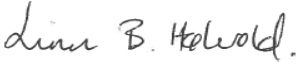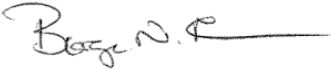only interpretations.**

(Friedrich Nietzsche)

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CONTRIBUTORS

	Paper I	Paper II
1. Planning and design	BNF, LBH, JB	LBH, BNF
2. Data gathering, analysis and interpretation	BNF, LBH	LBH
3. Manuscript preparation	BNF	LBH
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5. Approval of final manuscript version	BNF	LBH
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    Linn Benjaminsen Hølvold

    Tromsø, December 2012

LIST OF PAPERS

PAPER I

Optimization of formulation variables to increase antigen entrapment in PLGA particles

B. N. Fredriksen, L. B. Hølvold, J. Bøgwald, R. A. Dalmo

Polymer-Plastics Technology and Engineering 2012 October; 51(14): 1468-1473

PAPER II

Transgene and immune gene expression following intramuscular injection of Atlantic salmon (*Salmo salar* L.) with DNA-releasing PLGA nano- and microparticles

L. B. Hølvold, B. N. Fredriksen, J. Bøgwald, R. A. Dalmo

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SUMMARY

The most widely applied vaccines in salmon aquaculture today are based on water-in-oil formulations that provide excellent antigen depots, protect the antigen from degradation and induce strong inflammatory responses. Unfortunately, these vaccines show little efficiency against intracellular pathogens and can also cause side-effects such as autoimmunity and lesions at the injection site. In the search for efficient and biocompatible vaccine adjuvants, considerable attention has been given to the biodegradable copolymer PLGA (poly-(D,L-lactic-co-glycolic)-acid) and its potential use for the construction of injectable particles. The present work explored the use of such particles for intramuscular delivery of a plasmid DNA in Atlantic salmon (Salmo salar L.). The overall aim was to evaluate the influence of particle-use on the overall tissue distribution of the pDNA, transgene expression, innate proinflammatory and antiviral immune responses, expression of cytotoxic T-cell markers and injection site histopathology.

The first step was to establish a preparation protocol for PLGA particles to ensure consistent results in terms of size and encapsulation efficiency for the entrapment of a model antigen (Paper I). This protocol was later used to prepare pDNA-loaded PLGA nano- (~320 nm) and microparticles (~4 µm) for *in vivo* and *in vitro* use (Paper II). Tissue samples gathered over a period of 70 days showed similar distribution profiles for naked pDNA and pDNA encapsulated into PLGA nanoparticles. For microparticle-encapsulated pDNA the distribution profile highly resembled that obtained with the use of an oil adjuvant, demonstrating a potent depot at the injection site even at day 70. Encapsulated pDNA was able to induce expression of a luciferase reporter gene, but at lower levels compared to administration of naked or oil-adjuvanted pDNA. Immune responses were assessed by quantitative PCR over a period of 7 days. Particle formulations proved superior for the induction of the proinflammatory cytokine IL-1β, with little differences observed between pDNA-loaded and empty particles. PLGA nanoparticles carrying pDNA were the strongest inducers of antiviral responses, particularly in form of the Mx1 protein where significant levels of expression were observed in muscle tissue, spleen and head kidney samples. Histopathological

examinations of tissue samples from the injection site demonstrated strong inflammatory responses especially in samples from fish that had been injected with microparticle formulations. At day 30 post injection there appeared to be a chronic inflammation in the tissue similar to what was seen in fish injected with the oil adjuvant.

PLGA particles demonstrated central adjuvant properties following intramuscular administration of Atlantic salmon (Paper II), in the form of strong inflammatory responses as well as an ability to provide an injection site depot (microparticles). The use of nanoparticles was also found to induce innate antiviral responses that were not seen with naked or oil-adjuvanted pDNA and that could be beneficial to the immunogenicity of a viral vaccine. To better evaluate the potential of PLGA particles for delivery of DNA vaccines it will be necessary to conduct studies applying plasmids that encode immunogenic transgene proteins. Closer attention should also be paid to the inflammatory histopathology observed at the injection site, and any adverse effects this might have both for the health of the fish and the quality of the final consumer product.

A brief history of Norwegian aquaculture

Given the extremely long coastline that provides prime condition for sea-farming, it may perhaps seem natural that Norway today is one of the world's leading countries in salmonid aquaculture. The way leading to this point, however, has been long – and starts with the very first aquaculture more than 3000 years back[1]. The first documentation of aquaculture is from China as far back as 1122 B.C., although aquaculture likely started much earlier and may have developed independently at different corners of the world. Fish farming in ponds has been the dominant method for most of the aquaculture history, and was also the beginning of Norwegian aquaculture.

As early as the 1850s the first hatcheries for rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) were established for restocking purposes, with freshwater farming of rainbow trout for consumption initiated in 1910[2]. With government funding, the first attempts to raise rainbow trout in sea-water took place only a couple of years later, although the project was soon stopped due to poor profitability. For more than four decades the aquacultural activity was nearly non-existent, until farming started again around the 1960s[2]. Rainbow trout was long the main species in Norway, and was farmed in sea-water tanks. In 1969 there was just one farmer who raised Atlantic salmon in sea-water net pens, but the success of this experiment initiated an industry-wide transition. By 1977, Atlantic salmon had become the main species in Norwegian aquaculture, and the use of sea-water net pens was also defined as the standard farming technology[3]. From production figures for rainbow trout and Atlantic salmon of 433 and 98 metric tons, respectively, in the late 1960s[2], the official figures for 2010 showed a combined volume of nearly one million metric tons, of which Atlantic salmon accounted for about 95%[4]. A wide range of other fish species as well as crustaceans are farmed in addition to Atlantic salmon and rainbow trout, of which a few are Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), Arctic char (*Salvelinus alpinus alpinus*), blue mussels (*Mytilus edulis*) and noble crayfish (*Astacus astacus*)[2]. The total production volume of all species ranks Norway as number seven in the world in aquaculture output as of 2010, whereas only China ranks higher in terms of export of fish and fish products[5].

One of the greatest challenges in aquaculture has long been the mortalities and reduced production caused by a variety of infectious diseases. Cold water vibriosis, also

known as the 'Hitra disease', was a major problem in Norway in the 1980s, and sparked the initiation of the 'Healthy fish' research program. As a result the causative agent (bacterium *Vibrio salmonicida*[a]) was discovered and eventually a vaccine was developed, marking one of the economically most important achievements and contributions of aquaculture research[6]. Whereas most bacterial diseases are today kept under control through vaccination, intracellular bacteria and virus continue to cause problems for the aquaculture industry. The investigation of novel vaccine concepts is therefore of great importance, and requires an understanding not only of vaccinology but also of the immune system and its functions in fish. The following introduction aims to provide insight into the characteristics of fish immunology as well as some background on the use of vaccine delivery systems and DNA vaccination. Whereas much of what is known is based on experiments performed in mammalian species, references will be made to specific results obtained from research on different fish species.

Morphology of teleost immune organs

The morphology of teleost lymphoid organs varies between species, and whereas some have functional lymphoid tissues at hatching, most marine fish species with pelagic larvae are hatched with nearly non-existent lymphoid organs (reviewed[7]). The most important distinction between mammals and fish is that fish lack bone marrow and lymph nodes, which in mammals make up the primary lymphoid organs[7]. Instead, the thymus, anterior kidney (head kidney, or HK) and spleen are generally regarded as the major immune organs[8]. The thymus is the major site of T-cell lymphogenesis[9,10], whereas the HK holds the highest concentration of developing B-cells[11]. The HK lacks excretory tissue, but acts as a secondary lymphoid organ through the clearance of soluble and particulate antigens from the blood circulation by sinusoidal macrophages and endothelial cells[12,13]. Although the HK does contain low levels of antibody-secreting cells, the highest abundance of mature B-cells is found in the spleen[8,11,14]. Like the HK, the spleen also plays an important part in trapping blood-borne antigens[12]. Other lymphoid organs in

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[a] *Vibrio salmonicida* has later been renamed *Aliivibrio salmonicida*

fish are the mucosa-associated lymphoid tissues (MALTs), which include the novel interbranchial lymphoid tissue (ILT)[10,15].

Despite the differences between fish and mammalian species with regard to immune organs, fish possess morphological and/or functional equivalents of most innate and adaptive immune cells. The most central cells in innate cellular immunity are the phagocytic cells (macrophages and neutrophils) and non-specific cytotoxic cells (NCCs), although other granulocytes (eosinophils, basophils), natural killer-like (NK-like) cells and thrombocytes can also be found in fish (reviewed[16,17]). The existence of a dendritic cell (DC) equivalent in zebrafish (*Danio rerio*) was recently suggested and only this year a study reported on the functional identification of DCs in rainbow trout[18-20]. The main cells of adaptive cellular immunity are the T-cells, which act through recognition and response to peptide antigens associated with major histocompatibility complexes (MHCs) class I and II[16]. The first evidence of the existence of T-cells in teleosts dates back to the 1970s[21], whereas B-cells were first detected in the late 1960s through the discovery of immunoglobulin (Ig) in mucosal secretions[15].

A general introduction to fish immunology

The ability to withstand infection and eliminate invading pathogens is essential to all life-forms and is present to some degree in all multicellular organisms (reviewed[22,23]). With more than 23000 extant species, fish comprise one of the largest and most diverse animal phyla and also represent a major transition point in the evolution of immunity (reviewed[24]). Somewhere between jawless and jawed vertebrates there took place what is often referred to as 'the immunological big-bang', comprising two waves of gene duplication and the acquisition of recombination activator genes (RAG1 and RAG2), which gave rise to the adaptive immune system (reviewed[22,25]). Jawed fish are hence the earliest vertebrates known to possess not only a 'primitive' innate immune system, but also a more complex series of immune responses known as adaptive immunity.

Table 1 - Summary of the main components of the innate and adaptive immune system that have been found in teleosts (based on a selection of research and review papers[8,10,15,18])

Division	Component	Effector
Lymphoid tissues	Primary	Head kidney Thymus
	Secondary	Head kidney Spleen
	Others	Mucosa-associated lymphoid tissues (MALTs) Interbranchial lymphoid tissues (ILTs)
Innate components	Constitutive	Physical barriers; epithelial and mucosal linings of skin, gills and alimentary tract
	Cellular	Granulocytes Non-specific cytotoxic cells (NCCs) Monocytes/Macrophages Natural killer (NK)-like cells Neutrophils Dendritic cells (DCs)
	Humoral	Antimicrobial peptides (AMPs) Natural antibodies Complement system Other acute-phase proteins Cytokines Pattern recognition receptors (PRRs)
Adaptive components	Cellular	Cytotoxic (CD8$^+$) T-lymphocytes (CTLs) CD4$^+$ T-helper lymphocytes (T$_H$ cells) Cytokines
	Humoral	B-lymphocytes Antibodies Cytokines

The innate immune system is comprised of constitutive factors that are always present, and inducible factors that are subject to up-regulation during an immune response[23]. The constitutive factors are the physical barriers posed by the epithelial and mucosal linings of the skin, gills and alimentary tract, which also contain a large amount of the antibacterial enzyme lysozyme[23]. Lysozyme has a broader specter of activity in fish than it does in mammals, showing high efficiency towards both Gram-negative and Gram-positive bacteria, and has been shown to increase in response to infection long before a specific immune response can be mounted[23,26]. Innate immunity, although often referred to as non-specific, has through millions of years of evolution acquired a great efficiency for the recognition of structures that are highly conserved among a wide variety of pathogens. These structures, known as pathogen associated molecular patterns (PAMPs), are recognized by a series of pattern recognition receptors (PRRs) that are present on most immune cells as well as a few non-immune cells such as fibroblasts and epithelial cells (reviewed[12,27]). Pathogens that manage to breach the constitutive factors of innate immunity may be recognized and bound by PRRs to activate complement and cell signaling pathways, hence up-regulating the inducible innate immune factors[12]. Complement proteins have a variety of functions, including lytic, proinflammatory, chemotactic and opsonic activities, which ties them to non-specific phagocytic processes[12,26]. The activation of cell signaling pathways induces inflammatory mediators such as chemokines and cytokines. Cytokines act as immune response modulators and play and important part in in the development of adaptive responses, whereas chemokines are a superfamily of cytokines of which one of the most essential functions is the mediation immune effector cell-movement to sites of infection[12,28]. Two of the most important cytokines in mediating inflammatory responses are tumor-necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). Both are induced almost immediately upon infection and play key roles in the migration of effector cells to the site of infection[29], as well as contributing to the development of adaptive responses[30]. The discovery that human recombinant TNF-α elevated respiratory burst activity in rainbow trout macrophages indicated a conserved TNF-α receptor on trout leukocytes and provided evidence of a cytokine network that regulates immune responses in fish in a manner comparable to what is known from mammals[31]. The cytokine has later been cloned in a

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variety of species[32-34]. TNF-α has been shown to induce IL-1β in rainbow trout and enhance leukocyte migration *in vitro* as well as modulate the phagocytic responses of HK leukocytes[30]. Rainbow trout IL-1β was actually the first non-mammalian IL-1β sequence to be isolated[35], and has since been found in a number of species[36-40]. The existence of a second IL-1β gene has also been reported for the common carp (*Cyprinus carpio* L.)[41], as well as for rainbow trout and Atlantic salmon[42].

The distinction between innate and adaptive immunity, however common, is still an artificial one. Monocytes, macrophages, DCs and plasmacytoid DCs (pDCs) are known antigen presenting cells (APCs) in mammals (reviewed[43]), and provide an important bridge between innate and adaptive immunity through the presentation of pathogen-derived antigen to adaptive immune cells. Macrophages are so far regarded as the most important phagocytes and APCs in fish and are able to process exogenous antigen for presentation either by MHC class II, or by MHC class I following a delivery of the exogenous antigen to the cytoplasm (reviewed[44,45]). Along with B- and T-lymphocytes, RAG genes and memory formation, the MHC complexes make up the fundamental features of adaptive immunity, all of which are present in teleost fish[8,23]. Interestingly, the genome sequence of Atlantic cod has revealed what was long suspected, namely that the MHC class II gene has been lost from this species, along with the CD4 co-receptor[46].

The presentation of peptides by MHC class II results from an endosomal processing of exogenous antigen and enables the stimulation of CD4$^+$ T-cells (helper T-cells)[16]. Depending on the nature of the innate signaling pathways, these CD4$^+$ T-cells will differentiate into different subsets of effector cells of which the best defined are T-helper 1 (T$_H$1) and T-helper 2 (T$_H$2) cells (reviewed[47,48]). Cytokines play an important part in regulating the polarization of naïve CD4$^+$ T-cells, and in the absence of PRR mediated signaling molecules the T-cells themselves produce the cytokine IL-4 to drive T$_H$2 differentiation, promoting humoral immunity[48]. There are still uncertainties regarding the existence of T$_H$2 responses in fish[47], but the T-cell system shares many characteristics with its mammalian counterpart. It has also been suggested that the antibody repertoire of fish is less diverse than in mammals, with IgM long being the only known functional immunoglobulin in teleosts. However, more recent research has

revealed the existence of also IgD and IgT/Z (reviewed[15]). Macrophages and DCs, the primary responders to infection, provide both cytokines and co-stimulatory signals that promote the differentiation of T_H1 effector cells. IL-12 is regarded as the classic T_H1 promoting cytokine, but a range of other cytokines may also favor T_H1 differentiation, including type I interferons (IFNs)[48].

Healthy individuals of both fish and mammals express type I IFNs at low levels, but the expression will be further up-regulated upon stimulation of intracellular PRRs[49,50]. Despite the transient induction period even in the continued presence of an inducer, type I IFNs are the most important mediators of innate antiviral immune responses and contribute to inhibit viral replication, induce apoptosis to clear infected cells and also induce a wide variety of antiviral proteins (reviewed[49,51]). One of the most studied antiviral proteins is Mx1, a type I IFN-induced protein belonging to a subfamily of the large GTPases (reviewed[52]). As in mammals the expression of Mx in fish is generally up-regulated by IFN-signaling, although some degree of up-regulation may take place in response to infection without any IFN involvement[52,53]. As Mx will normally accumulate to much higher levels than IFNs and express for a much longer period of time, it can also be used as a tool for tracking the activity of type I IFNs[54]. Mx is thought to exert its antiviral function by interfering with viral replication, although not all Mx proteins possess antiviral activity[52]. The Mx1 protein has demonstrated antiviral activity against infectious pancreatic necrosis virus (IPNV) in Atlantic salmon, where it was found to be expressed exclusively in the cytoplasm[55]. Although this sets it apart slightly from Mx1 in most other species where the protein is expressed in the nucleus, it does coincide with cytoplasmic replication of IPNV[52]. Most fish species seem to express Mx solely in the cytoplasm[55,56], although studies in Atlantic halibut suggest expression in both the nucleus and the cytoplasm[57]. Just like type I IFNs the Mx protein seems to be up-regulated by infection rather than initiated by it, and has been shown to express at varying levels in (apparently) healthy Japanese flounder (*Paralichthys olivaceus*)[58].

Type I IFNs also provide an important link between innate and adaptive immunity through the up-regulation of MHC class I[59-61], and are critical for the clonal expansion of $CD8^+$ T-cells as well as for the generation of specific and non-specific memory cells[62,63]. Hence, whereas the differentiation of effector $CD8^+$ T-cells requires the specific binding

with antigen and MHC class I, the final destiny of the cells depends on exposure to type I IFNs, demonstrating the importance of both APCs and inflammatory stimuli for the development of adaptive immune responses.

The activation and differentiation of naïve CD8^{+} T-cells into effector cytotoxic T-lymphocytes (CTLs) through the processing and presentation of antigen peptides on MHC class I is an important aspect of the immune response to intracellular pathogens[63,64]. Non-specific cell mediated cytotoxicity (CMC) in fish is conducted by NCCs (suggested precursors of NK cells), NK-like cells and neutrophils, whereas adaptive CMC responses require MHC class I, CD8 and T-cell receptors (TcRs) (reviewed[65,66]). Sequences for CD8α and CD8β have been found in several species of teleost fish, including Atlantic salmon, rainbow trout and Japanese flounder, together encoding the ortholog of the CD8 co-receptor in mammalian T-cells[67-69]. Rainbow trout CD8α^{+} cells express CTL effector molecules and are found in high abundance especially in respiratory tissue, whereas the number in blood is not so high[70]. The study indicates that specific CMC in rainbow trout is predominantly executed by CD8α^{+} cells, with little contribution by CD8α[-]. Cytotoxic activity of CD8α^{+} cells has also been observed in ginbuna crucian carp (*Carassius auratus langsdorfii*) against allogeneic targets[71]. Although MHC class I is found on nearly every nucleated cell, the presentation by MHC class I on APCs appears to be more effective for the induction of CTL responses (reviewed[16,44]).

The differentiation and proliferation of CD8^{+} T-cells is mainly governed by the two T-box transcription factors T-bet and Eomesodermin (Eomes)[72,73]. T-bet is expressed and induced by T_H1 cells and was initially believed to be the only transcription factor vital to CD8^{+} T-cell differentiation and proliferation. It now appears that Eomes is able to uphold the effector functions of CTLs in the absence of T-bet, but as the transcription factors exert their influence at different stages of the differentiation they are both required in order to sustain the full range of effector functions[72-74]. Cells lacking both T-bet and Eomes instead differentiate into an IL-17 secreting lineage reminiscent of a helper T-cell fate implicated in autoimmunity and extracellular microbial defense[73,75].

The adaptive immune system plays a critical role in the protection against recurrent infections through the generation of memory cells as well as both soluble and membrane-bound antigen-specific receptors (reviewed[76]). Although teleost fish are in possession of an adaptive immune system, a high degree of pathogenic exposure from an early ontogenetic stage combined with an observed temperature-sensitivity of adaptive responses suggests that they rely more heavily on innate responses than mammals[8,23,77,78].

Mechanisms of cellular uptake

All mammalian cells are able to internalize fluids, molecules and particles by different mechanisms collectively referred to as endocytosis (reviewed[79]). The two main types of endocytosis are phagocytosis and pinocytosis, from which pinocytosis can be further divided into; 1) receptor mediated endocytosis (also known as clathrin-mediated endocytosis or CME), 2) caveolae-mediated endocytosis (CvME), 3) fluid phase endocytosis of bulk solutes (macropinocytosis) and 4) clathrin- and caveolae independent endocytosis (Fig. 2) (reviewed[80,81]). Phagocytosis is normally applied by professional APCs as a means of ingesting larger particles (0.5-10 µm) such as bacteria, and involves the recognition of PAMPs by PRRs on the surface of the phagocyte[79]. Pinocytosis, on the other hand, is a constitutive formation of vesicles that contain macromolecules and extracellular fluid. CME is commonly considered the 'classical route' of cellular entry and is carried out by almost every nucleated cell as a means of taking up essential nutrients. Prior to internalization, macromolecules are concentrated on the cell surface through receptor binding in clathrin-coated pits (CCPs)[81]. Caveolae are flask-shaped structures of 60-80 nm[82] that are especially abundant in muscle, endothelial cells, fibroblasts and adipocytes[81]. Caveolae vesicles have been reported as capable of bypassing lysosomes as well as internalize compounds much larger than the size of the caveolae, although the actual properties are still debated (reviewed[83]). Macropinocytosis involves the formation of transient membrane ruffles that protrude to engulf extracellular fluid and particles like bacteria, necrotic cells and viruses (reviewed[84]).

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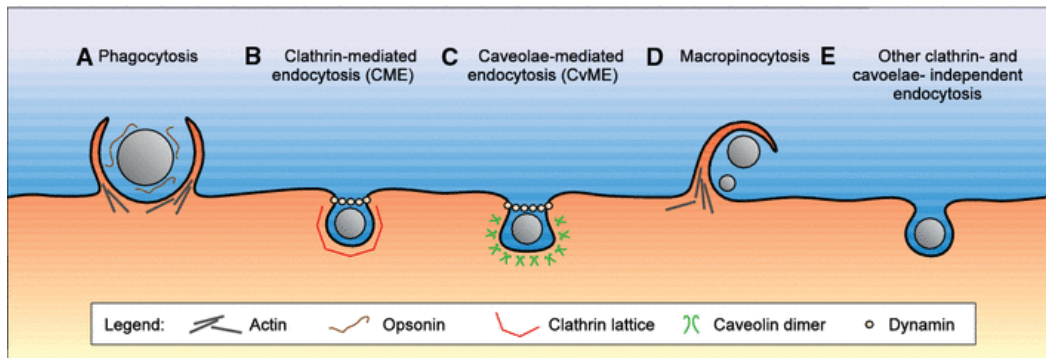
Figure 1 - Depiction of different endocytic pathways (figure from Hillarieau and Couvreur[85])

Endosomal sorting

Internalization by CME shows evidence of a pre-early endosome sorting process involving variations in adaptor proteins of the CCPs[86]. Subpopulations of CCPs internalize cargo destined for degradation, and transport it to a class of rapidly moving early endosomes on microtubules that mature quickly towards late endosomes. The majority of internalized material is delivered to a more static type of early endosomes that mature more slowly[86]. The recycling-ligand transferrin is non-selectively delivered to all early endosomes and therefore becomes more enriched in the static population[86].

Toll-like receptors (TLRs)

The most studied PRRs in fish are toll-like receptors (TLRs), a class of trans-membrane proteins that each is highly specific for a variety of pathogenic structures (reviewed[27,61]). A common way to classify the TLRs is by distinguishing between those that bind PAMPs at the cell surface, and those that recognize internalized PAMPs. While most TLRs are expressed on the cell surface, TLR 3, 7, 8 and 9 bind PAMPs exclusively in endosomal/lysosomal compartments and recognize agonists such as pathogen-derived nucleic acids[61,87,88]. The key features of TLR recognition and signaling appear to be highly conserved among vertebrates, and studies conducted in fish have found TLRs to respond to similar agonists and induce the expected cytokines[89,90].

A TLR that receives attention in this thesis is TLR9, one of the intracellular TLRs and recognizer of CpG motifs (described later) in bacterial deoxyribonucleic acid (DNA)[87]. Innate immune cells that recognize CpG motifs by TLR9 are DCs, monocytes, macrophages and neutrophils[91], but although TLR9 does exist in fish it is not yet clear whether it binds CpG motifs in a direct manner as observed in mammals[92]. It has, however, been shown that endosomal maturation of CpG-containing DNA (CpG-DNA) is necessary for TLR9 signaling in fish just as it is in mammals, where the process triggers a translocation of TLR9 from the endoplasmatic reticulum (ER) and Golgi apparatus to the endosomes[87,93-95]. TLR9 has been shown to up-regulate in response to stimulation with CpG-DNA in a variety of fish species, such as Atlantic salmon[96], rainbow trout[97] and Japanese flounder[98]. Rainbow trout and Japanese flounder both expressed TLR9 in spleen, HK, PBLs and gills, whereas expression was also found in the posterior kidney, heart and ovaries of Atlantic salmon. The protein sequence of Atlantic salmon TLR9 also revealed a conservation structural features that are crucial for signaling and adaptor functions in mammalian TLR9, further supporting the likelihood that both expression and function are conserved across teleost and mammalian lineages[96].

Vaccines – weapons of mass protection

In 1796 Edward Jenner was the first to conduct an immunization, steering the world down the path that would eventually lead to the eradication of small-pox[99]. The background for what can well be called a daring experiment was the discovery that milk-maids who had been exposed to the less pathogenic cow-pox rarely or never contracted small-pox. Using lymph from a pustule on the hand of milk-maid who was infected cow-pox, Jenner successfully immunized a boy so that later exposure to small-pox induced no signs of illness[99]. The closest method applied today is the live-attenuated vaccine, which is one of the five principle types along with; inactivated vaccines, subunit, toxoids and genetic based vaccines (reviewed[100]). The purpose of vaccination is ultimately to prime the immune system for specific pathogens by stimulating the formation of a long lived, antigen-specific memory cell population.

Vaccination in aquaculture

The existence of adaptive immune responses in fish makes them eligible for vaccination. The major breakthroughs in vaccines for farmed fish came with the introduction of adjuvants during the 1980s, but the first successful vaccination was carried out some 40 years earlier when Duff immunized cutthroat trout (*Oncorhynchus clarkii[b]*) with killed *Aeromonas salmonicida* and achieved protection against furunculosis upon challenge[101]. A brief overview over the developments in fish vaccinology, with emphasis on topics relevant to this thesis, is provided in table 2.

In the early stages of modern aquaculture, large amounts of antibiotics became the solution for combating emerging diseases. The use of antibiotics in Norwegian aquaculture reached its peak in 1987, with an accumulated weight of nearly 50 metric tons for the production of no more than 50 000 metric tons of fish[102]. The introduction of efficient oil-adjuvanted vaccines against bacterial diseases in the late 1980s meant a drastic reduction in the use of antibiotics. Today the annual production volume is about 1 million metric tons, whereas the use of antibiotics has been reduced by more than 99%[4].

The methods of vaccination applied in aquaculture vary depending on species, developmental stages and whether the fish are farmed in an enclosed environment or in sea-cages. The superior method for achieving protective immunity is injection, despite the fact that the need for anesthesia and handling subjects the fish to substantial stress[77]. Most injected vaccines are delivered intraperitoneally, whereas DNA vaccines are most efficient when injected into muscle[103,104]. Vaccination by immersion is a far more applicable method when the fish are small (<10g), and can be performed as dip vaccination or as spray, shower or bath (reviewed[77]). Oral vaccination is so far inferior to both injection and immersion, but if made effective it would require the least amount of labor as well as virtually no stress for the fish.

Among the greatest challenges in today's aquaculture industry is the lack of efficient vaccination strategies for combating diseases caused by intracellular pathogens. Conventional vaccines are generally strong inducers of humoral immune responses and have proven efficient against bacterial diseases that originally caused enormous losses to the industry[77]. However, they are largely incapable of inducing the immune responses

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[b] Previously named *Salmo clarkii*

necessary to resist infection by virus and intracellular bacteria. Live attenuated vaccines and DNA vaccines have shown great potential for inducing cellular responses (T_H1, CTLs) (reviewed[105,106]), but their use is limited due to risks of reversion to virulence (live attenuated) and potential chromosomal integration, amongst others (DNA vaccines). Hence, the need for strategies that will enhance cellular responses as well as maintain the general welfare of the fish is pressing.

Adjuvants and vaccine carriers

'Adjuvare' is latin and means to help or aid, which is exactly the purpose of adjuvants. And adjuvant is simply defined as a compound with the ability to increase and/or modulate the immunogenicity of an antigen[107]. Immune responses to vaccination are largely dependent on three signals[107]. The most central of these is the signal provided by the antigen (signal 1), which surveys the information required for a specific immune response. Signal 2 derives from the receptor-ligand interaction between APCs and T-cell antigens and is required in order to avoid aborted responses. The activation of APCs and orientation of the T_H response depends on signal 0, which is generally triggered through antigen recognition by PRRs.

The use of killed/inactivated pathogens or component vaccines suffers the drawback that both methods are poor inducers of adaptive immune responses, necessitating the use of adjuvants that can contribute to the immunogenicity of the vaccine[64]. The use of adjuvants dates back to the 1920s (reviewed[108]), but due to the slow progress in the field of fish vaccinology their potential in aquaculture was not fully appreciated until the introduction of oil-adjuvants in the late 1980s[109].

Developments in fish vaccinology		
↑	**2012**	
	2010	First PLGA-immunization by injection; intraperitoneal delivery of PLGA particles containing *A. salmonicida* membrane proteins[112].
	2008	First reported delivery (oral) of PLGA-encapsulated DNA to fish; study with Japanese flounder[113].
	2005	First licensed DNA vaccine for fish; Apex-IHN® for protection of salmonids against IHNV[114].
	1997	First use of an encapsulated vaccine; oral administration of *Vibrio anguillarum* encapsulated into alginate microspheres[115].
	1996	First use of PLGA particles in fish; oral intubation of Atlantic salmon with PLGA particles containing human gamma globulin (HGG)[116].
		First DNA vaccination of fish; rainbow trout was injected intramuscularly with a plasmid coding an IHNV antigen[117].
	1995	First commercial viral vaccine; Norvax® Protect-IPN was licensed in Norway[118].
	1981	First adjuvanted vaccine meant for injection and protection against *A. salmonicida* was licensed.
	1976	First licensed fish vaccine; orally administered killed *Yersinia ruckeri* to protect against enteric redmouth disease.
	1951	The (possibly) first report on viral immunization; intraperitoneal injection of carp with formalin-killed virus (likely spring viraemia virus).
	1942	First report of successful vaccination; oral administration of chloroform-killed *A. salmonicida* induced protection in cutthroat trout against furunculosis after challenge by injection or cohabitation[101].
		Intraperitoneal injection of killed or attenuated bacteria induced protection against *Aeromonas hydrophila* upon challenge.
	1938	Induction of protective immunity in fish after injection with killed *Aeromonas punctate*.
	1935	Heat-killed *V. anguillarum* induced a specific and temperature related agglutinin response after injection in eels.

The function of adjuvants

Adjuvants provide starting signals for and accelerate the immune response, and may also provide a functionally appropriate type of immune response (T_H1 vs. T_H2), increase the generation of (especially) memory T-cells and alter/modify the response in terms of specificity and breadth (reviewed[119,120]).

Adjuvants can be broadly categorized as particulate and non-particulate adjuvants. Particulate adjuvants owe at least some of their adjuvant properties to their microscopic particulate nature and generally exert their full potential when the immunogenic compound can be incorporated into or associated with the particle to ensure that antigen and adjuvant are distributed similarly and delivered to the same population of APCs (reviewed[121-123]). Many common adjuvants fall into this category, including water-in-oil emulsions, aluminium salts, immune-stimulating complexes (ISCOMs™), liposomes and various polymeric nano- and microparticles[121,123]. Non-particulate adjuvants generally exert immunomodulatory functions, and often benefit from an association with particulate adjuvants. The category of non-particulate adjuvants encompasses amongst others saponins, cytokines, TLR agonists (flagellin, Poly I:C, CpGs), Lipid A and carbohydrate polymers (e.g. β-glucan)[121].

Adjuvants have also been classified according to whether they act on signal 0, 1 or 2[107]. Most specific adjuvants, such as TLR agonists, act on signal 0 as well as indirectly on signal 2 through the activation of APCs and induction of cytokines and are classified as type A adjuvants. Type B adjuvants, comprising amongst others microspheres and some emulsions, exert their influence on signal 1 by enhancing antigen capture and presentation to T-cells by MHCs. As antigen presentation is not sufficient on its own, these adjuvants will require a co-delivery of immunostimulatory signals such as type A adjuvants. Finally, type C adjuvants are specific ligands of co-stimulatory molecules, with the ability to directly enhance signal 2.

The knowledge that adaptive immune responses require time to reach robust levels at low temperatures means that an efficient depot might be essential for the acquisition of protective responses in cold-water species such as Atlantic salmon[77]. The major success of mineral oil-adjuvants can largely be attributed to their depot effects, including an injection site depot with gradual vaccine release and a protection of the

vaccine against degradation[124]. Although oil adjuvants induce immediate and strong expression of pro-inflammatory signals in Atlantic salmon, the expression can be transient and difficulties concerning the administration of especially small volumes can lead to large variations between fish[50,77]. Oil-adjuvanted vaccines may also induce certain side effects, such as autoimmunity, chronic inflammations and adhesion of peritoneal organs to each other or to the cavity wall[125,126]. The perhaps most widely applied and efficient oil adjuvants for research purposes are Freund's complete (FCA) and Freund's incomplete (FIA) adjuvant, both composed of a mineral oil with surfactant and differing from each other only in that FCA contains heat-killed Mycobacteria[127]. Unfortunately, both may induce rather severe side effects and do not always provide a sufficient immunological response. Other mineral oil adjuvants, such Montanide™ and Alphaject, have been developed to maintain the advantageous effects seen with the Freund's adjuvants, but with a reduced risk of side effects[120]. Aluminum compounds have long been the most common adjuvants for human as well as veterinary vaccines and induce rapid and lasting antibody responses in addition to T_H2 responses through an enhanced uptake by APCs (reviewed[128,129]). Their exact mechanism of action is, however, still largely unknown and like oil adjuvants they are not optimal for the induction of CTLs. As a presentation of peptide by MHC class I is required for the activation of CTL responses, a good adjuvant for the induction of cellular immunity would be one that enables cytosolic antigen delivery as well as an up-regulation of T_H1 responses. ISCOMs™ as well as various polymeric nano- and microparticles provide good opportunities for targeting antigens to APCs and have also been shown to induce CTL responses (reviewed[130,131]).

The vaccination of aquatic animals offers challenges not only in terms of immunogenic factors such as temperature dependent immune responses and a lack of investigative tools for immune response research purposes, but also with regard to vaccination strategies. Given the high number of fish it is clear that repeated immunizations would be highly impractical at best. Aside from the need for vaccines with the ability to induce efficient cellular immunity, the use of efficient depot-creating adjuvants is therefore critical in order to achieve maximum protection.

Poly(D,L-lactic-co-glycolic)-acid (PLGA)

The potential of the biodegradable polymer PLGA for biomedical use has been the subject of research since the 1970s[132]. Ever since PLGA was approved for use in human and veterinary medicine by the American Food and Drug Administration (FDA), it has been applied for a wide range of products such as sutures[133], orthopedic fixtures[134] and as delivery matrices for pharmaceuticals[135]. The 1980s brought about the exploration of micro-sized (>1000 nm) PLGA particle constructs as carriers for delivery of vaccines and drugs[136-138], whereas a more recent development is the application of nano-sized (<1000 nm) particles with the intention of enhancing T_H1-responses to vaccination[139].

Qualities of PLGA

PLGA is a copolymer of lactic and glycolic acid that is synthesized by random ring-opening copolymerization of the cyclic dimers (1,4-dioxane-2,5-diones), where the monomeric units are linked together by ester linkages to create a linear, amorphous aliphatic polyester product[140] (Fig. 2). As a synthetic, thermoplastic polyester with a glass transition temperature (T_g) above 37˚C, PLGA has a chain structure that is sufficiently rigid to provide the mechanical strength necessary for formulation of drug delivery systems[141]. Amorphous DL-PLGA and DL-polylactic-acid (PLA) are generally used for the purposes of drug and antigen delivery as their polymer stereochemistry allows for a homogenous dispersion of antigens in the polymer matrixes[141]. The ability to vary the ratio of lactic to glycolic acid is, along with the high biocompatibility and non-toxic effect on biological systems[142,143], one of the key characteristics for the application of PLGA in vaccine delivery.

PLGA particle preparation

The most applied methods for preparation of PLGA particles are coacervation[144], spray-drying[145] and the double emulsion solvent-evaporation technique. Also known as water-in-oil-in-water, or $w_1/o/w_2$, the double emulsion solvent-evaporation method was first described by Ogawa et al. in 1988[137,146] and has since become the most frequently applied method for the encapsulation of antigens.

Table 3 - Process for preparation of PLGA particles by the double emulsion solvent evaporation method (w$_1$/o/w$_2$).

	Procedure
First water phase (W$_1$)	Antigen is dissolved in a 600 µl aqueous solution containing 0.2 % poly vinyl alcohol (PVA).

Oil phase (O)	PLGA is dissolved in 6 ml of the chosen organic solvent (5% w/v).

First emulsion (W$_1$/O)	The two solutions are emulsified by sonication, homogenization or magnetic stirring to create the first emulsion (w$_1$/o) of nano- or microsized droplets, commonly for durations of 20 sec to 5 min. The force and duration of this step especially influence antigen integrity and encapsulation.

Second water phase (W$_2$) **+** **Second emulsion (O/W$_2$)**	The first emulsion (w$_1$/o) is transferred to a second aqueous phase (w$_2$) containing 2 % PVA. Depending on applied force of emulsification (sonication/homogenization/stirring) this step may last for 1-10 min and is a critical determinant of the final particle size. The dispersion of droplets in the w-phase creates the water-in-oil-in-water (w$_1$/o/w$_2$) emulsion.

Solvent evaporation	The dispersion of droplets during the second emulsification contributes to the hardening of the particles by facilitating solvent extraction into the w-phase during solvent evaporation. Adding additional dH$_2$O will facilitate the process. During a stirring process lasting from 5-20 h (until the solvent is completely evaporated) the size of the particles is reduced through a packing of the polymer matrix that entraps antigens from the w-phase.

Washing	Resulting particles are washed 3 times in a stepwise process centrifugation process (500-25000 x g depending on particle size) in order to remove excess PVA from the particle surfaces.

Lyophilization	Washed particle suspensions are aliquoted to small containers and diluted with a lyoprotectant (1:3 in trehalose (5 mg/ml)). Aliquots are frozen at -80 ˚C, then freeze-dried at <0.01 hPa for a minimum of 48 h until all water is evaporated. The resulting product is stored in airtight containers at 4 ˚C.

Particle preparation by this method produces spherical particles with a negative surface charge due to the common use of polyvinyl alcohol (PVA) as a stabilizer[147]. Table 3 provides a general outline of the procedure. A selection of different formulation variables is presented in Paper I, whereas examples of specific formulation parameters can be found in Paper II.

The process of lyophilization creates pockets with high particle concentrations and can induce aggregation and an irreversible fusion of nanoparticles[148]. Crystallization might cause mechanical stress on nanoparticles and result in destabilization. Cryoprotectants and lyoprotectants prevent stress during freezing and drying, respectively. As such, both affect properties that may extend the shelf-life of the final product. Trehalose, both a cryo- and a lyoprotectant, is the most preferred for several reasons; low chemical reactivity, no internal hydrogen bonds which means it can form more flexible bonds with the nanoparticles, higher T_g and less hygroscopicity[148,149]. Stepwise washing is crucial for the removal of residual polymer, solvent and stabilizer from the final particle product, and also allows for a rough separation of particles by size. Some solvent and stabilizer will, however, always remain attached to the polymer.
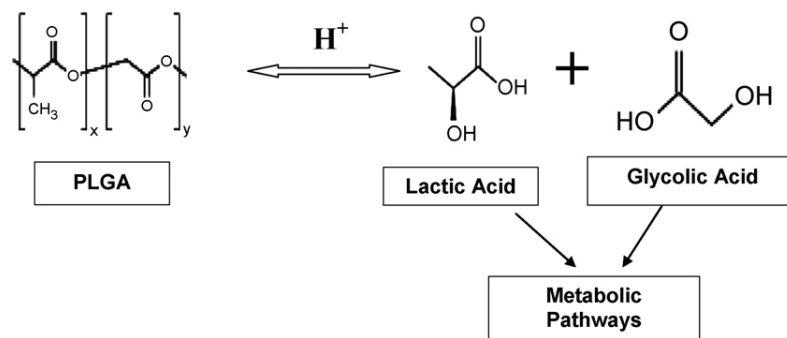
Figur 2 - The chemical structure and biodegradation products of poly(lactic-co-glycolic)acid. The ratio of lactic to glycolic acid is symbolized by the letters x and y, respectively (picture obtained from Kumari *et al.*[150])

Particle characteristics – traversing the maze

The $w_1/o/w_2$ preparation protocol offers a range of opportunities for tailoring the final particles characteristics to suit specific needs. Through the regulation of various aspects of the protocol, it is possible to influence characteristics such as size, porosity, antigen loading, zeta-(ζ)-potential (net surface charge) and degradation as well as antigen release. Although a protocol may be modified with the intention of altering just one of these characteristics, any change may influence other characteristics as well.

Size

Particle size is especially important with regards to tissue distribution and the potential and extent of cellular internalization. The parameter that exerts the most influence on particle size is the force applied during emulsification, and mainly during the second step of emulsification where the use of sonication has been shown to generate nanoparticles even when vortex had been applied for preparation of the first (w_1/o) emulsion[151]. Greater forces are required for the preparation of nanoparticles compared to microparticles, and sonication is often the method of choice for generating particles of small sizes[152]. Reports on the influence of molecular weight (M_w) on the resulting particle size are very variable. Most studies report low M_w PLGA as superior for the generation of small particles[152,153], although there have been studies conducted that suggest the opposite[154]. The use of stabilizers also affects particle size, and increased concentrations of PVA appear to result in smaller particles[147].

Porosity

Preparation of particles by the $w_1/o/w_2$ method has been reported to consistently create highly porous structures, irrespective of the final particle sizes[155,156]. However, it seems that minor modifications of the protocol may result in capsule structures with thin polymer walls[157]. The choice of temperature during the solvent evaporation process has also been shown to influence the uniformity of pore distribution. Evaporation at room temperatures resulted in particles with a thin, dense skin layer and a very uniform internal porosity, whereas low evaporation temperatures yielded slightly smaller particles with a thicker skin, where the pores at the center of the spheres were larger[156].

Encapsulation efficiencies and antigen loading

For most purposes of application a high encapsulation efficiency and drug loading is desirable. Whereas the second step of emulsification is the main factor influencing particle size, the first step affects the encapsulation efficiency and antigen integrity[151,158]. A study comparing homogenization and sonication for the preparation of microspheres found homogenization to favor higher encapsulation efficiencies as well as a linear release profile[159]. High M_w PLGA increases encapsulation efficiencies and therefore also loading, likely due to an increased viscosity of the oil-phase that results in a lower diffusion-rate of antigen/drug from the inner water-phase[154,160,161].

A wide variety of solvents have been applied for the preparation of PLGA nano- and microparticles. The rate of evaporation as well as the miscibility of the applied solvent with water is crucial for the resulting product, as a fast surface precipitation after emulsification is necessary to ensure a high encapsulation efficiency[162]. There are discrepancies in the literature with regards to the effect of different PVA concentrations on the efficiency of antigen/drug encapsulation, although there are indications that an increase in the concentration of PVA in the second emulsion enhances encapsulation[158], possibly by reducing the diffusion of antigen from the solidifying particles.

Zeta-(ζ)-potential

The zeta-potential of particles is of great importance as a positive (cationic) charge is known to enhance cellular internalization[154,163,164]. Residual surface PVA tends to create particles with a negative zeta-potential[147], but the amount of residual PVA will vary depending on other aspects of preparation. Sahoo *et al.* observed the least residual surface PVA when chloroform was applied as solvent compared to acetone and dichloromethane (DCM)[165], and also noted that PVA M_w as well as the concentration of PVA in the second water phase would influence the final residual amount. Surface PVA appears to be higher with smaller particles, raising the question of whether it is PVA that influences particle size or the other way around[166]. A decrease in polymer M_w also corresponds with a decrease in the polymer's T_g, while there has been observed an increase in zeta-potential[152,167].

Degradation and release characteristics of PLGA particles

By definition the degradation of polymers classified as biodegradable is at least mediated by a biological system[168]. The process of polymer erosion is, however, much more complex than mere degradation and will in addition to degradation depend on factors such as swelling, morphological changes and the dissolution and diffusion of oligomers and monomers[168]. PLGA degradation is hence the collective process of bulk diffusion, surface diffusion, bulk erosion and surface erosion. The addition of polymer constructs to a biological system will further increase the complexity of the process.

The degradation of PLGA is thought to take place by a bulk erosion mechanism[169,170] where the ester bond linkages in the polymer backbone are cleaved by spontaneous hydrolysis[171]. The first phase of degradation involves a decrease in the molecular weight of the polymer due to random and continuous hydrolytic ester cleavages, but demonstrates no actual loss of polymer mass[172]. During the second phase the acidic microenvironment generated by the process of hydrolysis acts to further degradation, and the polymer construct experiences a rapid loss of mass[172]. In large particles (>300 µm), the enhanced autocatalytic effect inside constructs influences the surrounding matrix, causing degradation rates to be higher inside particles than on the surface[173,174]. In the final stage of PLGA degradation the soluble oligomers created during the second phase are further fragmented to soluble monomers, resulting in a complete solubilization of the polymer (Fig. 2)[175]. A complete breakdown of lactic and glycolic acid takes place in the Kreb's cycle, after which a total removal occurs either through the respiratory route or via excretion by the kidneys or the liver[141,170,176].

The kinetics of degradation depends largely on PLGA M_w and composition[171], with an increase in molecular weight contributing to extend the period of degradation[175]. PLA (100:0) has the highest T_g and is the most hydrophobic composition, and polymers rich in lactide therefore demonstrate the slowest rates of degradation[141,167,171,177]. The amorphous nature of 50:50 co-polymers exempts them from this rule, and they show the fastest degradation rate[141,178]. The preparation of nanoparticles may cause a slight increase in the T_g of the PLGA[179], as will the molecular weight decrease in the polymer backbone during degradation[167]. Freeze drying the particles in presence of saccharides

such as trehalose yields high stability for storage, and refrigerator temperatures have also been shown to reduce the degradation to nearly undetectable levels[149,180].

Antigen release from PLGA particles

Irrespective of their size, PLGA particles prepared by the $w_1/o/w_2$ method demonstrate a bi-phased release profile where an initial burst release is followed by slow, continuous release[181]. The initial burst release observed shortly after particles have been dissolved in an aqueous solution is largely related to the type of encapsulated antigen, antigen concentration and polymer hydrophobicity[170]. The rapid release of antigen that is bound on or close to the surface is a result of antigen solubility as well as the penetration of water into the polymer matrix[166,170,182]. The second stage is a progressive release through a thicker, drug-depleted layer of polymer as well as through passages created by the continuous release of soluble oligo- and monomers from the degrading polymer[170]. Particle dimensions have been shown to influence the release of antigen during the first phase of degradation[180], whereas the continuous release phase has demonstrated almost identical rates of release for nano- and microparticles[166]. Release generally decreases with increased particle dimensions, possibly due to increased diffusion lengths coupled with a decreased surface-to-volume ratio[155]. Smaller particles have been shown to retain higher surface concentrations of PVA, which seems to contribute to a higher antigen release rate[158]. As the M_w of PLGA decreases there is an increase in degradation and hence also antigen release[146]. High shear forces may also generate more porous structures with higher burst releases[183].

Cellular uptake of PLGA particles

The cellular internalization of PLGA particles has been demonstrated with a variety of cell types both *in vivo* and *in vitro*. Depending on the size of the particles they can be internalized by macrophages/monocytes and DCs[184-186] by either by phagocytosis (0.5-10 μm), macropinocytosis (0.5-5 μm) or CME (<200 nm). Some studies also report on internalization by CvME, but although such studies will be described the uptake by caveolae of particles any larger than 100 nm remains a topic of debate[83]. The B-cells of

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fish have been shown to be phagocytic in a number of fish species, including Atlantic salmon[187,188], and phagocytic activity was recently demonstrated also in B-cells from mice[189,190]. Although 0.5 μm appears to be a general size limit for phagocytosis by B-cells, the uptake of polystyrene beads as large as 1 μm is observed both *in vitro* and *in vivo*[187]. Most nucleated cells can carry out uptake by CME, and myocytes have demonstrated an ability to internalize particles as large as 0.5 μm[122,191].

As the particle dimensions increase there is generally a reduction in uptake with regard to both rate and concentration[192,193]. However, studies conducted *in vitro* with rat macrophages report particle sizes of 2-3 μm as optimal for phagocytosis, demonstrating that the difference relates to the speed of attachment rather than internalization[186,194]. This could be attributed to the fact that pathogens commonly phagocytized by APCs are in the same size-range[194]. Human peripheral blood lymphocytes (PBLs) have demonstrated significantly higher uptake of 4.5 μm polystyrene particles compared to 1 μm particles, with noticeable differences also observed with regard to zeta potential especially for the smaller particles[164]. Cationic particles have been shown to internalize more readily than particles bearing a negative charge (anionic), offering an explanation as to the increased uptake often seen with high M_w PLGA compared to more anionic, low M_w PLGA particles[152,167,195]. Cationic particles also accumulate to a much higher concentration, whereas particles with no surface charge are the slowest to internalize[143,163,196]. CME and possibly also caveolae mediated endocytosis have been suggested as the dominant mechanisms for uptake of cationic particles in human PBLs, whereas a blocking of these routes lead to enhanced uptake by macropinocytosis[143]. Anionic particles appeared to internalize by mechanisms other than CME and caveolae[143]. Nanoparticles smaller than 200 nm seem to favor uptake by CME, although differences have been observed between cell lines[197]. Rejman *et al.* also reported a slow CvME as the main mechanism for uptake of 500 nm polystyrene particles[191]. In addition to zeta potential, a hydrophobic nature also works to enhance cellular uptake and may be a more critical characteristic than the surface charge[163]. Hydrophobic particles internalized more quickly than more hydrophilic particles in a study with mouse peritoneal macrophages, where particle diameters of 1-2 μm induced the largest uptake[196].

Studies comparing different incubation temperatures have found high temperatures (37˚C) to be favorable to particle uptake, while low or no uptake at all was been reported at 4˚C[195,198]. Temperature dependent endocytosis has also been demonstrated in salmon HK macrophage-like cells (SHK-1 cell line) for uptake of a fluid-phase marker, and although the endocytic process still continued 4˚C it was down to almost one third of the rate observed at 20˚C[199]. The time of incubation along with particle concentration also influence the efficiency of particle uptake, with a steady state of uptake generally achieved within 1-2 hours[185,193]. The efficiency of uptake has been shown to be higher at low particle concentrations, which is indicative of a saturable mechanism of internalization[193,200].

While the internalization of PLGA particles is relatively efficient, there is a continually ongoing exocytosis that takes place along with endocytosis, keeping up equilibrium between particle concentrations inside and outside the cell[192,201]. This process has been found to be energy-dependent, with internalization of small nanoparticles (<100 nm) observed after just one minute. When the extracellular nanoparticles were removed, 65% of the internalized particles were exocytosed within 30 minutes. The uptake was found to be concentration dependent at low concentrations, suggesting uptake by macropinocytosis[192].

Endosomal sorting and escape

Larger particles have been shown to require longer time to reach the late endosomes and lysosomes[191,202], and while both nano- and microparticles are capable of escaping into the cytosol, microparticles have sometimes been shown to remain in endosomes while some of their content is released to the cytosol[202]. The observation of PLGA nanoparticles in the center of early endosomes after internalization has been suggested as an indication of uptake by a non-specific mechanism such as macropinocytosis[201]. The localization may also relate to the physiological pH of early endosomes, in which the anionic particles are repelled by the negatively charged endosomal membrane. In late endosomes the acidic environment will interact with the PLGA and revert the surface charge to cationic[201]. This is an essential feature of PLGA particles, as it allows them to interact with the endosomal membrane, causing temporary

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and localized disruptions that release the particles into the cytosol[201,203]. Whereas the amount of particles that manage to escape into the cytosol may be as low as 15%, those that do so appear to remain inside the cells.

Biodistribution and depot effects of PLGA particle vaccines

Various particle qualities will determine the distribution following injection. Microparticles measuring more than 5-10 μm are generally unable to escape from the injection site as they are too large for efficient uptake by resident cells[186,204]. These particles will instead create a depot at the injection site, slowly releasing their content to the surroundings. Nanoparticles are small enough that they may move across biological barriers, or they can be transported as cargo inside cells (reviewed[205]). Nanoparticles that escape into the bloodstream will quickly be subjected to endocytic systems, with the final accumulation depending on the route of administration and the endocytic capacity of the given tissue or organ. Following intravenous delivery the clearance of particles is performed mainly by phagocytes in the liver or spleen[205]. Oral delivery has demonstrated a distribution of particles to liver, kidney, heart, brain, lungs and spleen in mice[142], whereas intramuscular injection has shown retention of microparticles in APCs in the lymph node[202].

The modification of surface charges as well as coating with various substances has been shown to alter the distribution profile as well as the specificity of cellular uptake of particles[205,206]. As hydrophobic particles are readily internalized by phagocytes, surface modifications that make the particles more hydrophilic may significantly reduce uptake and increase the time particles spend in circulation. Poly-(ethylene glycol) (PEG) is most commonly applied for this purpose, although PVA also contributes to a higher hydrophilicity[165,205].

Adjuvant properties of PLGA particles

The adjuvant properties of PLGA can be attributed to a broad range of functions. Particles can act as efficient delivery systems in order to enhance uptake by APCs[130], serve as depots for controlled release[176,207,208] and may protect the encapsulated antigen against degradation[130]. The suitability of inert polymer particles for sustained antigen release *in vivo* was first described by Preis and Langer more than three decades ago[209]. One of the advantages of PLGA is that it can be used for the encapsulation and/or absorption of a wide range of immunologic compounds, including immunostimulants such as β-glucan[210,211], TLR agonists like CpG DNA[212] and lipopolysaccharide (LPS)[213], recombinant proteins[139,195] and even whole viral particles[214], bacterial vaccines[215] and plasmid DNA (pDNA)[113].

An addition of empty PLGA microspheres to monocytes/macrophage cultures may be enough to achieve a certain level of activation[163]. Empty nano- and microparticles of PLGA have also been shown to induce TNF-α and IL-1β after uptake in APCs[139,216,217], with the necessary second stimulus perhaps derived from endogenous sources such as damage associated molecular pattern molecules (DAMPs) that induce a secretion of cytokines by macrophages. The addition of antigens or TLR agonists will generally enhance responses by stimulating the production of other cytokines such as IL-6 and IL-12[139], as well as prolong and enhance the presentation of antigen[203,208].

Despite a vast number of reported studies, the data on the influence of particle size on the induced immune responses is still highly debated[130]. The large variation in the sizes of particles reported for immunological studies is only one of the factors that make it difficult to pinpoint immune responses relating to size. In a study conducted with polystyrene beads (all measuring less than 150 nm) conjugated with ovalbumin, a decrease in size was found to correspond with a noticeable increase in T_H1-biased responses[218]. The general consensus still appears to be that nanoparticles favor the induction of cellular responses (T_H1 and CTL), while microparticles mainly promote T_H2 responses and the production of antibodies[122,207]. In a study by Kanchan *et al.*, nanoparticles measuring 200-600 nm were efficiently taken up by macrophages to induce IFN-γ and MHC class I as well as an antibody isotype favoring T_H1 responses[207]. On the other hand, microparticles (2-8 μm) were found to attach to the surface of APCs,

releasing their content there to induce a strong antibody response as well as an up-regulation of MHC class II and IL-4. Larger particles (20-50 μm) also attached to the surface of macrophages, but induced lower levels of antibodies than the smaller microparticles[207]. The attachment of relatively large PLGA particles (6.5±3.9 μm) to the surface of macrophages was also reported by Nicolete *et al.*, and they also found the particles to internalize with time[219]. Whereas nanoparticles demonstrated a less negative zeta potential and internalized more readily, the microparticles eventually induced a much more potent up-regulation of TNF-α and IL-1β.

The co-encapsulation of a model antigen with an immunostimulating adjuvant into PLGA microspheres has been shown to enhance the immune response of a particle vaccine[220], and a co-encapsulation rather than just co-inoculation of antigen together with a TLR agonist (e.g. CpG) seems necessary for the induction of potent CTL responses[221,222]. Although up-regulations of MHC class I and CTL responses are generally attributed to nanoparticles, the encapsulation of antigen into microspheres has been shown to up-regulate the expression of MHC class I as well as class II[208,223]. Particles measuring more than 500 nm mainly internalize by phagocytosis or macropinocytosis[218] and have in fact been shown as most efficient for the shuttling of exogenous antigen to the cross-presentation pathway[224]. The use of PLGA particles for antigen delivery increases the escape of antigen from endosomes to the cytosol[201,203,222], as well as the efficiency of presentation by MHC class I[212]. This has even been observed in B-cells where cross-presentation was otherwise not observed[203], and might be due to the increased retention time in early endosomes that has been observed with microparticles compared to nanoparticles[191,202]. As PLGA particles are only able to escape from endosomes and not lysosomes, a longer retention time in endosomes might provide increased opportunity for particles or their released antigens to translocate to the cytosol. Human macrophages derived from PBLs have been shown to present encapsulated exogenous antigen to T-cells *in vitro*, and the efficiency of presentations correlated well with the level of phagocytosis[223]. Following on, it is suggested that the use of PLGA nanoparticles for antigen delivery reduces the amount of antigen necessary to achieve the desired response[139].

PLGA studies in fish

Particles of PLGA have been extensively studied for their potential as carriers for vaccine and antigen delivery, with an increasing number of studies done also in fish. Oral immunizations have been performed with rainbow trout[225,226], Atlantic salmon[116] and Japanese flounder[113,227], whereas intraperitoneal delivery studies have been conducted in Atlantic salmon[210,211,214], Indian major carp (*Labeo rohita*)[112] and kelp grouper (*Epinephelus bruneus*)[228,229].

Transfection and reporter gene studies

Transfection is the deliberate introduction of nucleic acids to cells, and a plasmid DNA is commonly applied for this purpose. Plasmids are circular, double stranded (ds) DNAs with the ability to replicate autonomously in prokaryotes. Along with the gene of interest (GOI), plasmids that are used in gene delivery studies commonly contain promoter and enhancer sequences, a polyadenylation sequence, transcriptional termination sequence, an antibiotic resistance gene and an origin of replication (reviewed[230]). Transcription of the plasmid and translation of its messenger-ribonucleic acid (mRNA) to protein are both conducted by the cell's own apparatus in order to express the GOI.

Reporter genes are easily detectable and not normally expressed by the organism of study and are valuable tools for investigating factors that may influence gene delivery, such as; route of administration[103], administrated volume (reviewed[231]), pDNA dose[232], size of the animal[104], age of the animal[232] and experimental temperatures[78], as well as for assessing the potential of a species as candidate for gene therapy and/or DNA vaccination. Among the most frequently used reporter genes are luciferase (Luc), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) and β-galactosidase (β-gal)[230]. The potential of luciferase as a reporter gene was first reported more than two decades ago, and it has since become one of the most widely applied reporter genes in transfection studies[233]. The first reporter gene study in fish was reported Hansen *et al.* in 1991[232], just one year after Wolff *et al.* published their paper on the expression of a foreign protein following direct injection of a naked pDNA into mouse

muscle[234]. The expression of foreign genes so far appears to be higher in fish than in mammalian species for a given dose of pDNA, but levels of expression appear to vary greatly between individuals just as reported from studies on mice[235-237].

DNA vaccines and vaccination

The definition of DNA vaccination as provided by The Norwegian Biotechnology Advisory Board[238] is "the intentional transfer of genetic material (DNA or RNA) to somatic cells for the purpose of influencing the immune system". This sets it slightly apart from gene therapy, which in the same report is referred to as an introduction of foreign gene for purposes other than influencing the immune system. The mechanism of a DNA vaccine can in many ways be likened to that of a virus, as it requires the same cellular machinery in order to replicate and also triggers immune responses normally seen with viral infections[239]. Unlike conventional viral vaccines based on subunits or killed virus, a DNA vaccine may conserve the structure and hence also antigenicity of a transgenic antigen/protein[230,240].

Immune responses to DNA vaccination

A vital attribute of DNA vaccines is the ability to induce all three arms of adaptive immunity, namely; helper T-cells, CTLs and antibodies, although they were initially investigated in the attempt to find ways of delivering antigen to MHC class I and induce T_H1 responses[106,241]. Professional APCs are the cells that primarily contribute to the immune responses to DNA vaccination. Direct transfection of DCs provides the most efficient priming of naïve CTLs, and is perhaps the major mechanism for priming of these cells[242]. APCs are also able to take up exogenous antigens and process them for presentation by either MHC class II, or MHC class I following transfer of the antigen to the cytosol (cross-priming)[44,45]. These responses are vital in the cellular immune response following DNA vaccination.

The immunogenicity of DNA vaccines stems not only from the expression of the GOI, but also from properties of the plasmid vectors themselves. The ability of foreign nucleic acids to induce IFNs in chicken and mouse fibroblasts was discovered by two

independent research groups as early as in 1963[243,244]. The most studied pDNA property in terms of possible adjuvant effects is CpG motifs, regions in the DNA where a cytosine nucleotide occurs next to a guanine nucleotide and the two are linked by a phosphodiester bond. In vertebrates these sequences are highly methylated, whereas they show a much lower methylation frequency in viral and bacterial DNA (such as pDNA)[94,245]. These CpG motifs can act as PAMPs and are recognized as danger signals by the vertebrate immune system, resulting in a release of cytokines, macrophage activation, a differentiation of T_H1 effector cells as well as B-cell proliferation and secretion of antibodies[87,94,246,247]. The covalent linking of CpG-DNA to ovalbumin (OVA) has also been shown to enhance both uptake and cross-presentation of the antigen in B-cells[248]. The non-specific uptake of CpG followed by endosomal maturation are essential processes for signaling in APCs[88,249], and the induced cytokine profile appears to depend on whether CpG is recognized within early or late endosomes. Whereas detection of DNA-structures in early endosomes favors the induction of IFN-α, the sensing of linear DNA structures in late endosomes or lysosomes primarily leads to a production of IL-6 and TNF-α as well as DC maturation[250]. The immune responses triggered by CpG motifs demonstrate how the interaction of TLR9 with foreign DNA may bridge innate and adaptive immunity[251,252].

CpG motifs were long thought to be the sole contributors to the inherent adjuvant effects of DNA vaccines, but it now appears as if the basal adjuvant effects may in fact be independent of TLR signaling[253,254]. The escape of dsDNA into the cytosol enables the induction of cellular responses by different pathways and through different regulatory mechanisms[255,256], and it has been shown that TLR9-deficient mice are still able to mount significant T_H1 and B-cell responses *in vivo*[253,256,257]. The DNA-dependent activator of IFN-regulatory factors (DAI) was the first possible cytoplasmic DNA receptor reported and binds B-form (right-handed helical structure) dsDNA to enhance association with the signaling molecule TBK1, inducing IL-1β and type I IFNs as well as an activation of fibroblasts, macrophages and DCs[254,258,259]. Signaling by type I IFNs is indispensable for solid DNA vaccine-induced immunogenicity, but although it would seem that TBK1 rather than TLR9 signaling is essential for DNA vaccine-induced T-cell responses[257], a

co-injection of TBK-1 encoding plasmids to act as adjuvants with a DNA vaccine improved only humoral responses, not cellular[254].

DNA vaccination of fish

The first DNA vaccination of fish took place in 1996, when Anderson *et al.* immunized rainbow trout against infectious hematopoietic necrosis virus (IHNV)[117]. Since then several trials have been performed for a wide variety of fish species and pathogens and in 2005 a vaccine against IHNV infection in salmonids (Apex-IHN®, Novartis Animal Health) was also one of the first DNA vaccine ever to be cleared for marketing (by the Canadian Food Inspection Agency). In 1999 the injection of Atlantic salmon with pCMV4-G (plasmid-encoded glycoprotein) from a rainbow trout IHNV isolate induced significant protection against challenges with IHNV, even though the salmon were much larger than the rainbow trout in previous challenge studies[260]. DNA vaccination of fish has been shown to induce both innate and adaptive immune responses similar to what is seen in mammalian species, and seems especially efficient against rhabdoviruses (like viral haemorrhagic septicaemia virus (VHSV) and IHNV)[230,261]. These are simple RNA viruses with either five or six genes and a single viral surface protein (glycoprotein, or G protein) that acts as the protective antigen[230,262]. Other RNA viruses, or the larger DNA viruses, often offer more difficulty in identifying a protective antigen, although viral surface protein genes are almost always chosen for DNA vaccines[230]. Some pathogens, such as infectious pancreatic necrosis virus (IPNV), primarily cause disease in fry. Whereas vaccination by injection is highly impractical at this stage[77], the vaccination of post-smolts has been shown to induce protection upon challenge[263]. Good protection did, however, require the use of plasmids encoding all the large poly-proteins of the IPNV.

Early responses following DNA vaccination against rhabdoviruses largely indicate a systemic activation of the innate type I IFN antiviral pathway. Significant up-regulations of Mx and other virus-induced genes (VIGs) have been observed especially at the site of injection, although expression in spleen, liver and kidney is also commonly found[264-266]. In rainbow trout an immunization against VHSV enables the induction of cell-mediated immune responses encompassing both CTLs and NK cells and has also

been shown to significantly reduce the replication of virus during challenge[261,267]. Interestingly, when Cuesta *et al.* looked at the innate and adaptive responses in vaccinated and control fish after challenge, they found the highest increase in vaccinated fish to be that of innate immune responses[261]. The importance of innate responses in early antiviral defense was demonstrated by Lorenzen *et al.*, who subjected rainbow trout to VHSV challenge following vaccination with pIHN-G (plasmid-encoded IHNV glycoprotein). Whereas protection at late stages of the challenge could only be conferred by previous immunization with pVHS-G, the two vaccines induced similar levels of immune responses and protection during the first week following challenge[268].

The first dual DNA vaccination of rainbow trout against VHSV and IHNV was carried out by Boudinot *et al.* in 1995 by combining two plasmid vaccines in one injection[269]. The immunization efficiently induced Mx at the injection site as well as a production of antibodies equal to that obtained with individual vaccinations, thereby demonstrating the ability of the teleost immune system to accommodate multiple antigens. A more recent study applied the same procedure and reported that transfected cells expressed the G-protein of both vaccines[270]. When subjected to viral challenge the rainbow trout demonstrated a relative percent survival (RPS) at d80 post vaccination statistically equal to that obtained for individual vaccines and challenges (85-90%).

It is known that temperature is a critical parameter when it comes to immune responses in fish. However, a vaccination study with the VHSV G-protein in rainbow trout demonstrated that even though the involvement of innate and adaptive responses differed depending on temperature, DNA vaccination still conferred protection at temperatures ranging from 5-15˚C[78]. The delayed onset of adaptive responses at the low temperature range was effectively compensated for by a prolonged protection by innate antiviral mechanisms, supporting the consensus that fish rely more heavily on their innate defense repertoire than mammals[26].

It is not yet known whether teleost TLR9 binds pDNA the same way it does in mammals[92]. The administration of CpG-containing DNA to fish does, however, induce similar immune responses to those observed in mammals, including macrophage activation, leukocyte proliferation and expression of cytokines such as IL-1β and IFN-α[95,98,271-273]. The administration of CpG oligodeoxynucleotides (ODNs) as stand-alone

prophylactic agents has also been shown to enhance the survival of rainbow trout following bacterial challenge[91], as well as induce protective antiviral immune responses such as Mx to reduce viral titers and mortality upon challenge of Atlantic salmon with IPNV[273]. The expression of reporter genes is not only higher in fish, but also seems to have a longer duration. Transgene expression has been detected at the injection site as long as two years after injection of glass catfish (*Kryptopterus bicirrhus*)[274], and Tonheim *et al.* detected both supercoiled (sc) DNA and luciferase expression at the injection site 535 days after intramuscular injection of Atlantic salmon with pDNA[275].

Administration and distribution of DNA vaccines

Intramuscular injection

Intramuscular injection is widely applied for pDNA delivery in fish and generally results in strong expressions of transgene at the injection site[103,117,232]. Studies in mice have found the dispersion of pDNA immediately following intramuscular injection to take place primarily between the muscle body and epimysium (connective tissue that ensheaths the entire muscle)[276]. Myocytes and mononuclear cells take up pDNA after administration[277], but despite a rapid initiation of uptake the subsequent uptake is slow and cells along the muscle fibers have been shown to be transfected over a period of hours following injection[276,278]. A patch-like distribution in muscle tissue has been reported following injection of ink, suggesting the hydrostatic pressure caused by rapid injection of high volumes causes the fibers to 'spread', thus allowing for unequal distribution[237,277]. With very small fish this initial dispersion of a vaccine might be enough to ensure the perfusion of intact pDNA to more distant tissues, while in large fish the injected volume will mainly rest along the needle trajectory[269,279,280]. The transportation of pDNA to and from blood to other tissues has been reported for various fish species[117,280-282]. Plasmids have been recovered from sites such as liver, spleen, head-kidney, heart and intestine for some time after injection[235,282], but mainly persist at the site of injection[275,280]. Degradation of the pDNA starts within five minutes following injection of mice, with as much as 95-99% of the initial pDNA amount degraded within

90 minutes[236,283]. The rate of degradation in the tissue of cold-water fish remains to be determined.

The extent of histopathological changes at the injection site following intramuscular DNA delivery in fish appear to increase with an increase in vaccination dose[267,280], but vaccination will generally induce only moderate local tissue damage in form of degeneration of myocytes, haemorrhages and a transient influx of inflammatory cells[279].

Other routes of delivery

Other routes of pDNA administration that have been investigated in fish are intravenous (i.v)[282,284,285], intraperitoneal (i.p)[235], particle bombardment[103] and oral delivery[286]. Accumulation of naked pDNA took place primarily in the heart, kidney and liver following i.v administration, whereas oral delivery resulted in a recovery of DNA fragments from the pyloric region, mid and distal intestine and blood, as well as kidney and liver.

Uptake of plasmid DNA

A wide variety of mammalian cell types has been shown to take up pDNA (reviewed[287]), but so far uptake in fish has only been reported for myocytes[269], head kidney macrophages[285] and endocardial endothelial cells (EECs)[282]. The exact mechanisms by which myocytes take up pDNA remain to be determined, but several suggestions have been made[287]. It was previously theorized that direct injections caused temporary membrane disruptions and/or pores that allowed for the entry of pDNA[232], but studies have shown that such disruptions in fact work to abolish transfection[237]. Another mechanisms that has been proposed is entry through the T-tubular system of myofibers[288]. Danko and Wolff also suggested that the multiple nuclei found in skeletal muscle fibers might enable foreign DNA to persist, with the T-tubules and caveolae being other, unique muscle structures that likely enable DNA uptake[289]. In 1989, two independent studies reported on the involvement of specific receptors in the cellular uptake of ODNs and DNA, finding the process to be saturable as well as size dependent[290,291]. The hypothesis uptake of DNA by receptor-mediated endocytosis has

been strengthened by a number of other studies and is generally considered to be the main mechanism of uptake[278,287,292].

Of the number of cell-surface receptors investigated in terms of DNA binding and uptake, scavenger receptors (SRs) in particular have been a subject of interest. These receptors comprise a broad family of membrane proteins capable of binding a wide range of anionic ligands and are present on several different cell types (reviewed[293,294]). The uptake of pDNA by SRs has been demonstrated in Atlantic cod atrial EECs[282], but although the SRs may bind DNA they appear not to be essential for the immunostimulatory activity of CpG DNA[295].

Inside the cell

The uptake of pDNA can take place via different endocytic pathways, including macropinocytosis[296]. Following uptake by phagocytosis or receptor-mediated endocytosis the vesicles carrying pDNA will fuse with and release their content into endosomes[79]. At this point any receptors will dissociate due to the slightly acidic pH, while the bulk volume is destined for degradation in late endosomes or lysosomes[80]. Whereas the mechanism of transport from early to late endosomes is not known, the subsequent delivery to lysosomes is thought to occur by fusion and has also been observed with macropinosomes[297]. For pDNA to be able to transfect the cell it needs to escape full degradation, but the exact mechanism by which it escapes macropinosomes, endosomes and/or lysosomes is not yet known (reviewed[298]).

Once in the cytoplasm the movement of pDNA to the nucleus is restricted by the mesh-like structure of the actin cytoskeleton[299], which increases the exposure to degradation by cytosolic nucleases[300]. It has, however, been suggested that pDNA is transported through the cytoplasm by means of the microtubule network[301]. The final obstacle before entry into the nucleus is the nuclear envelope (reviewed[302]). Molecules smaller than ~40 kDa may diffuse passively through the nuclear pore complex (NPC), which forms channels across the nuclear envelope. Larger molecules are transported to the nucleus by an energy dependent process that requires the presence of a nuclear localization signal (NLS) (reviewed[303,304]). Nuclear uptake of pDNA has also been suggested to take place during cell division as the nuclear envelope disassembles[305].

Factors influencing transfection and transgene expression

Nucleic acids have a poor intrinsic transfection efficiency due to their large size and negative charge (reviewed[306]). The processes of gene transcription and translation are carried out by the cell's own machinery, and may be influenced by a variety of factors such as pDNA vector design, pDNA concentrations, administration volumes, age and size of the fish, water temperatures and method as well as route of administration.

It has been well documented that the DNA topoform has a strong influence on the efficiency of transfection[307,308]. Supercoiled DNA is frequently reported as the most potent topoform[113,308] followed by open circular (oc) forms, whereas linearization of the DNA has been shown to nearly abolish the expression of transgene and linearized pDNA[236,307]. The choice of promoter also has a profound influence on the level of transgene expression[237,309] and the cytomegalovirus (CMV) promoter has often been reported as the most effective[236,310]. Because of this, the CMV promoter is the most commonly applied in gene transfection studies and its potency has been demonstrated also in fish[117,240]. The use of an intron and polyadenylation (termination) signals further improves expression[309].

Despite the inherent adjuvant effects of DNA vaccines, it has been shown that immune responses triggered by DNA vaccination may in fact limit the duration of transgene expression[309]. The lack of anti-Luc antibodies means that both levels and duration of expression are commonly higher in studies applying luciferase compared to more immunogenic antigens, and immune responses have only been observed with the application of large pDNA doses and potent adjuvants such as FCA[311]. A co-administration of vectors encoding Luc and immunogenic proteins (β-gal, G protein) decreases both the strength and duration of luciferase expression, as the initiated antigen specific cytotoxic responses work to eliminate transfected cells expressing the transgenic protein[240,311]. The stimulation of PRRs may also induce responses that can be detrimental to transgene expression. The hallmark cytokines of the inflammatory response, TNF-α and IL-1β, have both been shown to inhibit transgene expression *in vitro* and *in vivo*[312,313], as have the T_H1 associated cytokines IFN-γ and IFN-α[313,314]. Levels of inhibition have been shown to correlate with the levels of CpG-induced cytokines, and synergistic effects have also been observed[312,313]. The inhibitory effect takes place at the

mRNA (post-transcriptional) level, hence not causing vector degradation, inhibition of total cellular protein synthesis or elimination of infected/transfected cells[313,314]. The contribution of CpG motifs has been asserted by showing that methylation of plasmid vectors before administration, or pre-administration of anti-cytokine antibodies, increased transgene expression[313,315].

Whereas intramuscular injection is widely acknowledged as the superior method of administration to achieve high levels of transgene expression[103,104,235], the result still depends on factors such as dose, volume and fish size and age[104]. Transgene expression has been found to be higher in young and growing fish[232], and small fish sizes appear to favor not only the distribution of pDNA throughout tissue but the distribution of transgene expression as well. Expression of luciferase in thymus, gills, spleen and kidney has been reported for small fish (<5 g)[240,280], although the highest expression is consistently detected at the site of injection in myocytes, infiltrating cells and epithelial cells lining small capillaries[240,269,279]. This highly restricted location of expression has also been observed after intramuscular injection of a LucZ gene in mice, where as little as 6% of the myofiber cells were found to be transfected and 70% of these were located in the same area[277]. Massaging the tissue around the injection site immediately following injection reduces transgene expression, likely as a result of the vaccine being forced out of the tissue[237]. The first reporter gene study in fish indicated the existence of a maximum above which there would be no further increase in expression[232]. This observation is supported by later findings in zebrafish and rainbow trout[240], and there are indications that excessive DNA concentrations may actually reduce transgene expression[103]. The injection of large volumes might contribute to a spatial distribution by creating temporary gaps between fibers[277], which appears to induce higher expression as well as reduce the variations commonly observed in *in vivo* transfection studies[231,237]. A pre-injection of an isotonic solution created the same effect, and lead to both higher and more equal transgene expression among individuals after DNA injection[277]. The observation that DNA slowly accumulates in cells over time refuted the theory that hydrostatic pressure during injection facilitates uptake[278], but goes well with the observation of reduced variations as a result of an increased spatial distribution.

Advantages , disadvantages and challenges of DNA vaccines

The potency of DNA vaccines for inducing the different branches of both innate and adaptive immunity has already been described. DNA vaccines also show high efficiency when given at early life stages[316,317] and provide the benefit of inducing protective immunity over a wide span of temperatures[78]. Vaccination can be successfully carried out without the need of conventional adjuvants and generally cause low degrees of tissue damage compared to what is often observed with intraperitoneally injected, oil adjuvanted vaccines[126,318]. The advantages of DNA vaccination still stretch beyond merely the immunological capacities. Looking at the concept from a manufacturer's or investor's standpoint, DNA vaccines are relatively inexpensive and easy to produce. The processes required for production are identical for all DNA vaccines, and the ease of cloning also enables rapid modifications in a way that is generally not obtainable with conventional vaccine preparations[231]. The ease of cloning also provides the potential for multivalent vaccines through the cloning of multiple gene segments into one plasmid vector, although multivalent vaccinations by a simple mixing of DNA vaccines have already proven efficient[268,269].

The histopathological side effects induced by DNA vaccines may be less than what is seen with today's commercial vaccines, but there is still a range of potential side effects to the fish. Antibody responses to host DNA could potentially be induced as a result of the adjuvant effects posed by the foreign DNA and cause autoimmunity, or the immature immune system of neonatal individuals might recognize the encoded transgene product as a self-protein to induce a state of tolerance (reviewed[319]). Moreover, the uptake of pDNA by myocytes following intramuscular administration means these cells may be targeted by CTLs, which could result in autoimmune myositis (reviewed[320]). Although very rarely observed, there is always a risk that the pDNA will integrate into the chromosomal DNA, although neither autoimmunity nor integration has been observed during initial studies in goldfish[320,321]. With the processes of DNA uptake and expression still not fully understood and given the high rates of degradation, relatively large doses of DNA are required in order to achieve sufficient levels of expression. An increase in dose will often bring about an increase in histopathological changes at the injection site, as well as an increase in inflammatory responses that may not always be desirable[280]. To

overcome these issues more effort needs to be put into gaining understanding of the mechanisms of pDNA-uptake, from the moment of administration until the stage of transcription and translation in the nucleus. There is considerable research being done on the application of carrier systems and/or DNA-complexes to facilitate entry and reduce degradation[192,201] but without a deeper understanding of the underlying processes the optimization of such systems remains difficult. Although transfection levels are often low, several studies have demonstrated a persistence of transgene expression at the injection site that might coincide with a time when the fish would normally be ready for slaughter[275]. There have been experiments with suicidal DNA vaccines for fish, where the plasmid vector includes a protein to induce apoptosis after an immune response has been triggered[322].

The use of DNA vaccines for species like Atlantic salmon introduces the risk of environmental release. Plasmids may leak from the administration site or be secreted through faeces after uptake by intestinal bacteria, and with the persistence of pDNA residues already demonstrated in Atlantic salmon[275,323] it could be transferred by consumption of vaccinated animals. For further reading, there are numerous review-papers published that address the advantages and disadvantages of DNA vaccines[230,231,324,325].

PLGA particles as carrier systems for DNA vaccines – focus on fish

The potential of PLGA particles as adjuvants and carriers for DNA vaccine delivery has received considerable attention in mammalian studies[157,201,326]. In spite of this, reports on the use of PLGA particles for DNA delivery to fish are nearly non-existent. In 2008 Tian *et al.* were the first to report on the use of PLGA microcapsules containing a plasmid vaccine for the oral immunization of Japanese flounder against lymphocystis disease virus (LCDV)[113]. Following immunization they detected transgene expression in gills, intestine, spleen and kidney from fish vaccinated with encapsulated pDNA. The encapsulated pDNA also induced higher levels of antibodies compared to control fish injected with naked pDNA. Tian and Yu later demonstrated a significant increase in resistance to LCDV infection after oral administration of a pDNA vaccine

encapsulated into PLGA nanoparticles[227]. Finally, the last study so far reported was by Adomako *et al.*, who used PLGA nanoparticles incorporated into feed for oral delivery of a DNA vaccine against IHNV to rainbow trout[226]. They found that particles were mostly taken up in the hind gut, but that a significant proportion of fish that showed uptake in gastrointestinal epithelial cells did not demonstrate detectable levels of transgene expression. Although antibody responses could be detected in fish given high doses of pDNA, the RPS at a six weeks post-vaccination challenge was still only 22%.

Harikrishnan *et al.* performed an intraperitoneal injection of kelp grouper against *Vibrio alginolyticus*, using PLGA-liposome encapsulated ODNs containing unmethylated CpG-sequences[229]. The administration of encapsulated ODNs induced significant antibody responses compared to naked ODNs and also provided a somewhat higher protection against challenge. Prior to the work presented in this thesis, no reports have, however, been made on the injection of PLGA encapsulated DNA vaccines in fish.

DNA-loaded PLGA particles

The $w_1/o/w_2$ method described previously is frequently used for the encapsulation of pDNA into PLGA particles, but results are highly variable with regard to encapsulation efficiency and loading as well as DNA degradation and release. The hydrophilic nature of DNA complicates the process of encapsulation as it increases the risk of plasmid diffusion into the w_2 phase during solvent evaporation[327], and the encapsulation process also offers multiple challenges in terms of preservation of bioactivity. While purified pDNA in solution is very stable compared to other vaccine compounds, the process of particle preparation involves several steps that can be detrimental to DNA integrity. Reports on the integrity of pDNA following encapsulation range from drastic reductions in the content of SC DNA, to nearly total conservation of the SC topoform[113,201]. Among the factors known to affect the integrity of the encapsulated pDNA are the polymer composition and M_w, shear force, preparation temperatures, solvents and the concentration and M_w of the applied stabilizer (commonly PVA)[160,328,329]. The primary cause of DNA degradation during preparation of particles is the shear forces applied during the emulsification processes. For pDNA in solution sonication for as little as five seconds can be enough to reduce the SC content by nearly

50%[330], whereas a decrease in SC DNA content from 89% to 65% as reported by Tian *et al.* seems common following particle preparation[227]. Lowering shear forces generally increases particle size, but may reduce the degeneration of pDNA[113]. The M_w of the PLGA is one of the main factors influencing the preservation of pDNA integrity during sonication, and despite some inconsistencies in the literature it seems that polymers with a high M_w offer the most protection[328]. The results have often been attributed to the increased viscosity of the oil-phase that is obtained when applying high M_w PLGA.

Polymers with a high M_w also result in the highest encapsulation efficiencies and lower the burst release of pDNA[154,160,161,181,331], which is consistent with the PLGA qualities previously described. Also consistent with previous descriptions is the observation that small particles promote a faster release of pDNA[227]. DNA and PVA both act as mild anti-plasticizing agents, particularly when PLGA of high M_w is applied[332]. After a short time of incubation and degradation an increase in the T_g of polymer at the particle surface creates a form of surface film, which works to slow the release of pDNA. No significant increase in T_g has, however, been observed when pDNA is encapsulated into a low M_w PLGA. Although the process of lyophilization may nearly abolish the transfectivity of DNA, this can be restored after an addition of mono- and disaccharides[333]. The addition of carbohydrates to the DNA-containing water-phase during preparation has also been shown to increase encapsulation as well as reduce DNA nicking during lyophilization[329].

Transgene expression by PLGA-encapsulated pDNA

Encapsulated DNA has been shown to be more potent than naked DNA at mediating transgene expression *in vitro* in a variety of cells types[160,334,335]. However, *in vivo* studies report on a superiority of naked DNA in eliciting transgene expression not only compared to PLGA particles but other formulated DNA vaccines as well[235,334]. There can be many explanations for the reduced transfection efficiency observed *in vivo* when applying encapsulated DNA vaccines. A large reduction in the content of scDNA will lead to a reduced transfectivity[236], and the gradual release that is provided by PLGA particles could also result in an availability of pDNA that is too low to elicit an effective response[334]. Continuous exocytosis of particles may be another limiting factor to

transgene expression following administration of encapsulated DNA[201]. It is, however, possible that this ongoing exocytosis is actually beneficial to the resulting transgene expression as it might release particles to more distant cells[192].

Small particles (<100 nm) are often shown to internalize more rapidly, and also show the highest transfection efficiencies[336]. PLGA particles of low M_w generally induce higher transgene expression levels, possibly due to a higher zeta-potential that facilitates uptake[152]. However, even though nanoparticles can escape endosomes to release pDNA in the cytosol, an *in vitro* experiment using non-PLGA cationic polymers and liposomes reports that neither total nor released amount of intracellular DNA correlates with transgene expression[337]. It is suggested that the positive charges lead to an interaction with negatively charged nuclear molecules such as ribosomal RNA (rRNA), thus inhibiting transcription and translation[337,338]. The adsorption of pDNA onto the surface of PLGA particles, rather than encapsulation, has been shown to result in a higher transgene expression, but the expression also declined more rapidly[335].

Immune responses following administration of PLGA-encapsulated pDNA

In addition to the proinflammatory cytokines induced by empty PLGA nano- and microparticles (IL-1β and TNF-α)[216,217], the use of different particle sizes might influence the resulting cytokine profile after administration of encapsulated or particle-bound CpG DNA[339]. In addition to proinflammatory cytokines, nanoparticles have been shown to also enable an induction of antiviral cytokines such as type I IFNs in addition[339]. The encapsulation of pDNA encoding antigens has also been shown to elicit CTL-responses, even with pDNA-amounts that elicited no such responses after naked administration[340,341], Moreover, encapsulated pDNA also enhanced the total antibody response at high doses, while inducing a more rapid and complete seroconversion when lower doses were applied[341].

The injection of PLG microspheres into muscle has been shown to result in a foreign body response, with a large influx of different inflammatory cells that appear largely related to microspheres especially at later time-points[331]. These infiltrating cells were also the ones that were primarily transfected, an observation similar to that made in other studies[269,326].

Other particles in vaccine delivery to fish

The extensive use of PLGA as vaccine delivery vehicles can largely be attributed to their high biocompatibility as well as the ease with which the particles can be prepared. Other compounds have, however, also been investigated as carrier systems for vaccination of fish (Table 4), either on their own or in combination.

Table 4 - Compounds tested as vaccine delivery systems in fish species.

Compound	Which is?	Mode of carriage	Species
Alginate	Polysaccharide from brown algae	Encapsulation	Carp[115] Rainbow trout[115,342] Japanese flounder[343]
Chitosan	N-acetyl-D-glucosamine copolymer, derived from crustacean shells	Encapsulation	Nile tilapia (*Oreochromis niloticus*)[344,345] Japanese flounder[346] Asian sea bass (*Lates calcifer*)[347]
Liposomes	Artificial lipid bilayer vesicles	Encapsulation	Carp[348,349] Kelp grouper[350]
Poly(ε-caprolactone) PCL	Biodegradable, synthetic polymer	Coating	Indian major carp[351]
Calcium phosphate	Inorganic, biodegradable and biocompatible material	Coating	Indian major carp[352]

Current challenges in the use of PLGA particles as carriers/adjuvants

Despite a growing number of optimistic reports on the adjuvant/carrier properties of PLGA particles, there are still many hindrances to be overcome. One of these is the preparation protocols, where different drugs/vaccines require different conditions. While some compounds are easily encapsulated, others – like DNA vaccines – are more difficult to encapsulate in an efficient manner. Low encapsulation efficiencies not only result in low antigen loading, but also mean that a large amount of drug/vaccine goes to waste during particle preparation. The detrimental effects that encapsulation may have on certain antigens such as pDNA are also major limiting factors at the moment, and need to be resolved. Particle preparation is also low-scale work as of yet, with considerable effort to be made before the process is optimized on a large scale suitable for mass-production.

Concerns and precautions regarding PLGA nano- and microparticles

Any construct/compound, when brought down to sub-micron sizes, will exhibit new and potentially harmful characteristics[353]. The small size means they can interact with biological membranes in an entirely new way, thus inducing responses not seen with larger constructs. As with all new applications it takes time to fully survey the potential side-effects. *In vitro* studies using PLGA nano- and microparticles have so far not revealed any toxic effects, not even at large doses[160,200]. The various concerns regarding the use of PLGA particles in aquaculture vaccines were recently reviewed by Nielsen *et al.*[354].

AIMS OF STUDY

For the last 20 years there has been considerable effort devoted to the application of DNA vaccines as well as the use of PLGA particle constructs as vaccine carriers. Whereas the aquaculture industry has largely managed to control the outbreak of bacterial diseases through conventional vaccination methods, the battle against viral diseases still rages. As conventional DNA vaccination has offered promising results against viral pathogens, any method for enhancing the efficiency of such vaccines is of great value. This study looked to evaluate the potential of PLGA particle delivery constructs as a means of improving the responses to DNA vaccination in fish.

The specific objectives were to:

- Establish a protocol for the preparation and characterization of PLGA nano- and microparticles for purposes of research.

- Investigate the effect that encapsulation of pDNA into PLGA nano- or microparticles would have on tissue distribution and persistence following intramuscular injection of Atlantic salmon.

- Investigate the effect that encapsulation of pDNA into PLGA nano- or microparticles would have on tissue distribution and expression of a luciferase reporter gene following intramuscular injection of Atlantic salmon.

- Evaluate innate immune responses and the expression of cytotoxic T-cell markers after intramuscular injection of PLGA nano- and microparticles carrying pDNA.

ABSTRACTS

Paper I

Optimization of formulation variables to increase antigen entrapment in PLGA particles.

Efficient antigen entrapment is a key factor in preparation of poly (lactide-co-glycolide) acid (PLGA) vaccine formulations when the antigen is of short supply. This study presents a systematic approach in the testing of formulation variables with the objective to increase antigen entrapment in particles when the antigen stock concentration was low. Some of the experimental variables tested were poly (vinyl) alcohol (PVA) concentration in the inner (W_1) and outer (W_2) aqueous phase, W_1/oil (O) phase ratio and choice of organic solvent. The double emulsion solvent evaporation technique was applied to prepare PLGA particles with sonication as the emulsifying force. To measure antigen entrapment efficiency, the antigen (bovine serum albumin, BSA) was isotope labeled with ^{125}iodine (^{125}I). Our results demonstrated that a low PVA concentration in the inner aqueous (W_1) phase was beneficial to achieve a high encapsulation efficiency of antigen. On the contrary, in the outer aqueous (W_2) phase, a high PVA concentration favored antigen entrapment. We also demonstrated that decreasing the W_1 to O/polymer ratio contributed to increased entrapment efficiency. Testing different organic solvents (ethyl acetate, dichloromethane and chloroform), either alone or in combination, revealed that using chloroform as solvent resulted in the highest encapsulation of antigen and the highest production yield. Some of the results presented in this work are in disagreement with well-established formulation variables from previous studies.

Paper II

Transgene and immune gene expression following intramuscular injection of Atlantic salmon (*Salmo salar* L.) with DNA-releasing PLGA nano- and microparticles.

The use of poly-(D,L-lactic-*co*-glycolic) acid (PLGA) particles as carriers for DNA delivery has received considerable attention in mammalian studies. DNA vaccination of fish has been shown to elicit durable transgene expression, but no reports exist on intramuscular administration of PLGA-encapsulated plasmid DNA (pDNA). We injected Atlantic salmon (*Salmo salar* L.) intramuscularly with a plasmid vector containing a luciferase (*Photinus pyralis*) reporter gene as a) naked pDNA, b) encapsulated into PLGA nano- (~320nm) or microparticles (~4μm), c) in an oil-based formulation, or with empty particles of both sizes. The ability of the different pDNA-treatments to induce transgene expression was analyzed through a 70-day experimental period. Anatomical distribution patterns and depot effects were determined by tracking isotope labeled pDNA. Muscle, head kidney and spleen from all treatment groups were analyzed for proinflammatory cytokines (TNFα, IL-1β), antiviral genes (IFN-α, Mx) and cytotoxic T-cell markers (CD8, Eomes) at mRNA transcription levels at days 1, 2, 4 and 7. Histopathological examinations were performed on injection-site samples from days 2, 7 and 30. Injection of either naked pDNA or the oil-formulation was superior to particle treatments for inducing transgene expression at early time-points. Empty particles of both sizes were able to induce proinflammatory immune responses as well as degenerative and inflammatory pathology at the injection site. Microparticles demonstrated injection-site depots and an inflammatory pathology comparable to the oil-based formulation. In comparison, the distribution of NP-encapsulated pDNA resembled that of naked pDNA, although encapsulation into NPs significantly elevated the expression of antiviral genes in all tissues. Together the results indicate that while naked pDNA is most efficient for inducing transgene expression, the encapsulation of pDNA into NPs strongly up-regulates antiviral responses that could be of benefit to DNA vaccination.

GENERAL RESULTS AND DISCUSSION

The need for new and more efficient vaccines against present and emerging viral diseases in aquaculture is indisputable, and much effort is being put into the development of strategies to enhance vaccine efficacy. One method that has proved more promising in fish than in mammals is DNA vaccination, although satisfactory results have so far mostly been obtained for rather simple structured viruses such as rhabdoviruses. Polymeric particles as delivery systems for DNA vaccines and/or antigens have long been a focus of extensive research in mammalian systems, with PLGA being one of the most investigated polymer constructs. However, despite the need for more sophisticated DNA delivery-methods there have so far only been three studies on PLGA-encapsulated DNA vaccines in fish, and a mere nine studies in total that look at PLGA particles for antigen delivery. Beyond establishing a protocol for the preparation of PLGA nano- and microparticles a central point of this work was therefore to evaluate the effect that the encapsulation of pDNA would have not only on transgene expression, but also the tissue distribution and retention of pDNA, proinflammatory and antiviral responses and injection site histopathology.

Establishing a protocol for particle preparation

Of the three most established protocols for preparation of PLGA particles, the double emulsion solvent-evaporation method ($w_1/o/w_2$) is the most widely applied for encapsulation of a wide variety of drugs and antigens[139,212-214]. This was therefore the protocol of choice for the studies presented in this thesis. However, it soon became clear that previously reported studies on the preparation of PLGA particles often lacked details and could at several occasions not be replicated in our lab. In order to achieve particles with both the intended sizes and encapsulation efficiencies when working with low antigen concentrations it therefore became necessary to investigate some of the most central process variables, including, 1) choice of organic solvent, 2) PVA concentrations in the internal and external water phases and 3) volume ratios. This work is presented and discussed in paper I, whereas a general description of the $w_1/o/w_2$ method is given in table 3 (p. 26).

Using sonication as the emulsifying force and bovine serum albumin (BSA) as a model antigen it was found that chloroform as solvent resulted in both the highest production yield and a higher encapsulation efficiency compared to ethyl acetate, dichloromethane or solvent-combinations. It was also shown that alterations of PVA concentrations exerted great influence on the entrapment of antigen. Low concentrations of PVA in the internal water phase lead to an increase in encapsulation efficiencies, as did an increase of PVA concentrations in the external water phase. The results were used to establish a general preparation and encapsulation protocol that later proved successful for the entrapment of different model antigens, whole virus particles (IPNV) and to a lesser extent also β-glucan[210,211,214]. It was, however, much less efficient for the encapsulation of pDNA (Paper II), where encapsulation efficiencies and loading were low for both nanoparticles (~320 nm) and microparticles (~4 μm).

Despite the overall simplicity of the $w_1/o/w_2$ preparation protocol, Papers I and II together clearly demonstrate the importance of optimizing the preparation conditions to suit the antigen to be encapsulated. The application of PLGA with a higher molecular weight for pDNA encapsulation might provide increased protection against shear during particle preparation as well as increase encapsulation efficiencies[160,331]. Given the high influx of inflammatory cells observed at the injection site after administration of encapsulated pDNA it could be speculated that nanoparticles may not necessarily be optimal for internalization and subsequent transgene expression. APCs have been shown to readily internalize microparticles in a size-range comparable to those applied in Paper II (~4 μm)[194], and if the intention is merely to increase transgene expression the use of nanoparticles may hence not be required. As the integrity of encapsulated antigens depends largely on the first step of emulsification, while size is mainly determined by the force applied in the second step, preparation conditions could be altered to ensure maximum preservation of intact pDNA while still generating particles in a desirable size-range[151].

A problem with the determination of DNA integrity following encapsulation is that the methods for extracting pDNA may in themselves be detrimental, and a large amount of particles may be required to achieve suitable pDNA concentrations. Similarly, the determination of encapsulation efficiencies has largely been based on either the

extraction of pDNA from dissolved particles and/or indirectly through quantifying the amount of pDNA present in supernatant following encapsulation[160]. Both methods suffer from the influence of solvents and stabilizers which distort spectrophotometric readings even at low concentrations (unpublished results). Extraction of pDNA from particles may be incomplete, hence giving the impression of an encapsulation efficiency that is higher than in reality. The preparation protocol established in Paper I was hence based on the use of trace amounts of isotope-labeled antigen, which allowed for direct gamma readings from a known amount of particles.

An *in vitro* release study was conducted for Paper II in order to determine particle stability as well as the release of pDNA from the different particle constructs. It was found that both particle sizes demonstrated high burst releases followed by an extended lag phase where the particles still retained some pDNA after 70 days at the chosen temperature (8 ˚C). The burst release was considerably higher from nanoparticles and likely resulted from a high portion of surface associated pDNA[166]. Inherent qualities of pDNA such as a relatively large size and negative charge probably contributed to the results, as the charge in particular increases the risk of diffusion from the internal to the external water phase during solvent evaporation[327]. The comparatively large size of microparticles means a much longer diffusion path for centrally located pDNA than is the case with nanoparticles. A greater portion of the encapsulated pDNA might therefore be located within the central particle matrix rather than the surface or surface associated matrix, explaining the lower burst release observed with microparticles. The applied polymer was a low molecular weight PLGA with a fifty-fifty ratio of lactic to glycolic acid, qualities that have been reported to often result in poor encapsulation efficiencies as well as a high burst release[153,331]. The high burst release would have to be taken into consideration for later *in vivo* applications.

Tissue distribution profiles

Fish are known to take longer in initiating adaptive immune responses and especially at lowered temperatures[77,78]. Much of the success of the vaccines applied in aquaculture today has been attributed to their ability to create injection site depots[124], and it was therefore necessary to investigate the depot and distribution profile of PLGA particles following intramuscular injection (Paper II). In order to do this a fraction of the encapsulated pDNA was labeled with an iodine isotope [^{125}I], so that the gamma count could later be determined for collected tissues. The distribution of nano- and microparticles was compared to that of naked pDNA and pDNA emulsified with an oil-adjuvant (FIA). The distribution profiles for naked pDNA and nanoparticle-encapsulated pDNA were highly similar, which was expected given the high burst release observed from these particles during the *in vitro* study. The slightly higher retention at the injection site at day 70 still confirmed the previous observation that some pDNA remains associated with particles even at this time-point. Microparticles proved as efficient as the oil adjuvant at creating a depot at the injection site, in addition to reducing the differences in retention that were observed in the other group of fish. Some characteristics were observed in all groups, such as a rapid elimination of pDNA from the blood and accumulation in the kidney. Previous studies have also reported on the distribution of naked pDNA by blood following intramuscular delivery, as well as retention in kidney[235,281,285].

Injection site histopathology

The determination of histopathological changes at the injection site is an important preclinical assessment step in the evaluation of new vaccine strategies. Previous studies with DNA vaccines in fish have demonstrated that the histopathological changes at the injection site are usually minor and transient[279,280], but little information exists on tissue reactions to PLGA particles.

In Paper II samples of injection site tissue were examined for signs of haemorrhages, muscle degeneration and inflammation. Samples were chosen from time-points that would give some insight into both immediate and chronic histopathology.

Microparticles, with and without pDNA, were shown to induce strong inflammatory reactions that spread beyond the needle trajectory and that appeared to create a chronic inflammation at the injection site. The changes appeared more extensive in terms of both cell diversity and tissue distribution than what was seen in fish injected with the oil-formulation, and were reminiscent of a foreign body response to DNA-loaded PLG microspheres previously reported in mice[331]. Nanoparticles also induced considerable inflammatory reactions compared to administration of naked pDNA, although not as extensive as the microparticles. Although no effort was made to determine the nature of the inflammatory cells, it would seem that the use of PLGA particles might enhance the influx of APCs to the injection site. As APCs are important orchestrators of both innate and adaptive immune responses such a response could prove highly beneficial to DNA vaccine efficiency. However, the small number of samples means further research is necessary before any conclusions can be drawn.

Proinflammatory cytokines

The ability to induce innate immune responses is a central property of adjuvants. Despite being highly biocompatible, the ability of empty PLGA particles to induce the pro-inflammatory cytokines IL-1β and TNF-α has been previously described[216,217]. In Paper II the expression of these cytokines was quantified at the mRNA transcript level following administration of empty nano- and microparticles, naked pDNA, oil-adjuvanted pDNA and pDNA encapsulated into nano- and microparticles. Whereas the expression of TNF-α was generally low for all groups, the microencapsulated pDNA did seem more potent at up-regulating the expression in muscle, head kidney and spleen. It was found that particles of both sizes induced levels of IL-1β at the injection site far exceeding that observed with oil-adjuvanted pDNA, and that expression seemed to relate exclusively to particle size for the days that were investigated. Microparticles retained the highest level of expression at day 7 and the levels were by then significant even to those seen with nanoparticles.

Antiviral responses

The rationale for the very first research on DNA vaccination was to find a strategy that would enhance cellular immune responses in form of T_H1 and CTL[106], as conventional vaccines are generally potent inducers of primarily humoral responses. The reporter gene applied in Paper II is considered to be poorly immunogenic[311], but pDNA itself is able to induce immune responses through recognition by cytosolic receptors[254,258], or by recognition of CpG motifs by TLR9[87,247]. The antiviral responses were evaluated by quantifying the expression of IFN-α and the type I IFN-induced protein Mx. Whereas encapsulated pDNA significantly up-regulated the expression of IFN-α at day 1, later time-points demonstrated low expression and little variation between the different treatment groups. Mx is often used as a means of determining IFN-activity, as it expresses at much higher levels[54].

Transgene expression in Atlantic salmon

One of the primary motivations for encapsulating pDNA into PLGA particles prior to injection is to protect the plasmids from degradation by extracellular nucleases. DNA vaccination is a promising method for eliciting antiviral protection in aquacultured fish, but still suffers from drawbacks in form of low expression of transgene products that could be attributed to factors such as 1) rapid degradation of pDNA following administration, 2) rapid escape of pDNA from the injection site, 3) limited uptake of pDNA by myocytes and/or APCs, 4) low rates of endosomal escape[236,278,283,298]. PLGA particles have been shown protect antigens from degradation[130], create injection site depots[176], enhance uptake by different cell types[130] and increase antigen delivery to the cytosol[201]. Despite the superiority of direct intramuscular injection for pDNA delivery[103] there was, however, no reports on the intramuscular delivery of PLGA particles carrying pDNA into fish prior to the work presented in Paper II.

Comparing the intramuscular delivery of naked pDNA to pDNA in an oil-emulsion or encapsulated into either nano- or microparticles is was found that although encapsulated plasmids were able to induce transgene expression at the injection site at the mRNA level, the highest levels were observed with naked and oil-adjuvanted pDNA.

Consistent with the observations of tissue distribution the microparticles consistently induced slightly higher levels of transgene expression than nanoparticles. With an average size of ~4 μm, the microparticles may also have been in the optimal size range for internalization by APCs attracted to the injection site[194]. It was also observed that administration of naked pDNA and nano-encapsulated pDNA induced low levels of reporter gene transcripts in the head kidney, although the expression was negligible compared to what was found at the injection site.

It has been speculated that the slow release during the lag phase of PLGA particles may reduce transgene expression at it leaves very little pDNA available to transfect the cells[334]. However, this offers no explanation as to how naked pDNA and nanoparticle-encapsulated pDNA, with almost identical distribution profiles, can show such different potencies for inducing transgene expression. The same applies to the similarities in tissue retention seen with microparticles and oil-adjuvanted pDNA.

Although not addressed in Paper II, it is known that the process of particle preparation can exert some detrimental influence on pDNA integrity[113,201]. As the forces applied during emulsification are generally considered the primary source of shear, it would appear that the application of homogenization rather than sonication for the second step of microparticle formulation might have preserved a larger amount of intact pDNA, contributing to the induction of higher transgene expression levels. This could also have explained the levels of transgene expression observed with the oil-formulation, where only brief homogenization was applied to prepare the emulsion. However, even though the inferiority of encapsulated pDNA for eliciting transgene expression *in vivo* has been reported in mammalian studies[334], particle-delivery is commonly superior to naked pDNA for *in vitro* transfection[160,334]. This suggests the reduced transgene expression results from more than just a reduced amount of intact pDNA. Several studies have reported on the inhibitory effect of inflammatory responses on transgene expression, with the effect lasting for as long as the inhibitory cytokine is present and even reports of synergistic inhibitory effects of various cytokines[313,314]. As the administration of encapsulated pDNA did results in a very high expression of IL-1β, such an inhibitory effect might likely have influenced the expression of the reporter gene.

Further studies

The study presented in Paper II provides some insight into the influence of encapsulation on transgene expression, inflammatory and antiviral responses as well as injection site histopathology following intramuscular administration. It does not, however, offer any direct clarification as to whether or not encapsulated pDNA might enhance protection during a challenge study. It was shown that transgene expression levels are higher with naked pDNA and oil-formulations, but the use of a poorly immunogenic reporter gene means the study offers no insight into the potential benefits that enhanced proinflammatory and antiviral responses might exert on an adaptive immune response. IFN-α in particular is known to be one of the central players in innate responses to DNA vaccines, and amongst a range of other functions up-regulates the expression of MHC class I to enhance CTL responses[49,59]. As the encapsulation of pDNA into nanoparticles likely enhanced the expression of IFN-α in all tissues, as measured primarily through the quantitation of Mx expression, it seems reasonable to hypothesize that the use of an immunogenic antigen might have resulted in an increased expression of CD8$^+$ T-cells. Moreover, the inflammatory histopathology observed particularly with microparticles indicated an influx of APCs at the injection site. This could enhance the uptake of larger particles and possibly contribute to an increased CTL response, either by direct transfection of the cells, or through cross-presentation following uptake of protein secreted by nearby transfected cells.

What lies ahead?

The 'trial and error' approach has been frequently applied since the early days of vaccinology, and has undoubtedly brought about many good results with respect to disease prophylaxis. However, there are many pathogens towards which satisfactory results are not achieved with conventional strategies. DNA vaccination has long been a subject of research in fish, whereas the use of PLGA nano- and microparticles as delivery systems is still a very new concept for aquatic species. Both are still areas that require substantial amounts of research if the mechanisms underlying processes such as uptake and intracellular transport, and the factors influencing these, are to be understood in a

way that will allow for maximum optimization of vaccine constructs. While PLGA particles in theory do offer potential for the improvement of vaccine delivery, one should always be open to the observable facts in order to maintain a focus within the areas that actually yield satisfying *in vivo* results.

The development of a standardized protocol for the conduction of *in vitro* and *in vivo* experiments would ease the communication between researchers, as well as facilitate and perhaps speed up the research processes being carried out at different facilities. The study presented in Paper I was a direct result of the either lacking or contradictory information reported in previous studies, and laid the ground for all subsequent work in our laboratory. Following on, a detailed description of the particle preparation process was therefore provided in Paper II to allow for easy comparisons and (potentially) well-grounded criticism.

Apart from complicating the exchange of scientific results the lack of established and reproducible protocols makes it difficult to evaluate the potential of a large scale production of PLGA particle vaccines. Also, the topic of PLGA particle-application in aquatic animals has barely been touched upon, with the study presented in Paper II bringing the total of PLGA studies in fish (as of December 2012) up to no more than twelve. For the moment the focus should be on evaluating the use of PLGA particles not only in terms of vaccine efficacy, but perhaps more with respect to potential adverse effects both to the fish and the environment.

MAIN CONCLUSIONS

- PLGA nano- and microparticles both demonstrate low encapsulation efficiencies and a high burst release of pDNA
- PLGA nano- and microparticles with and without pDNA both induce strong proinflammatory responses at the injection site in Atlantic salmon
- PLGA nanoparticles carrying pDNA induce innate, antiviral responses in muscle, head kidney and spleen
- PLGA particles carrying pDNA are able to elicit transgene expression at the injection site as well as low levels in kidney, but are inferior to naked pDNA.
- PLGA nanoparticles demonstrate rapid escape from the injection site and a tissue distribution profile similar to that of naked pDNA
- PLGA microparticles provide injection site depots and a tissue distribution profile comparable to oil adjuvanted delivery

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