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PLGA and PLA particles as vaccine delivery systems for Atlantic salmon

A study on formulation and use with an emphasis on immune responses

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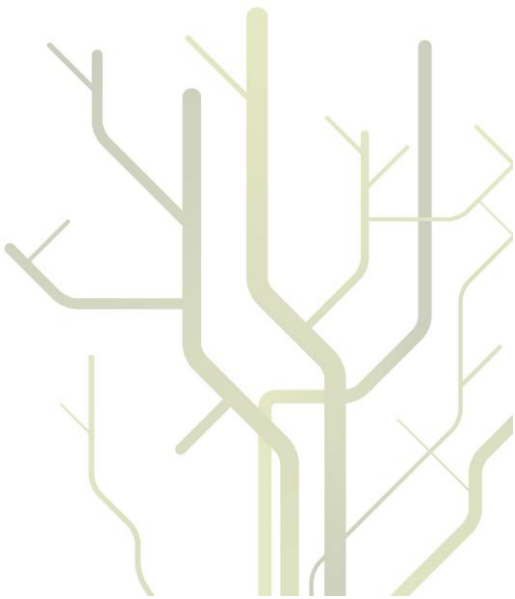
A dissertation for the degree of Philosophiae Doctor
January 2012



2.2.1. Preparation of particles

HBsAg loaded particles were prepared by w/o/w emulsification solvent evaporation method, this technique has been used extensively for encapsulation of many antigens including HBsAg (Shi et al, 2002; Yan et al, 1994). Briefly, PLA was dissolved in 4 ml dichloromethane (DCM). 0.75 ml HBsAg were mixed with 0.5 ml of aqueous solution containing 10% m/v PVA. The primary emulsion was formed by dispersing the two immiscible solutions using an ultra-turrax (T25 Janke & Kunkel, IKA-Labortechnik, ……) for 2 min at 24.000 rpm. This emulsion was added to 30ml of 2 % m/v PVA solution dropwise and homogenized for 10 min at 10.000 rpm with high shear homogeniser (Silverson L4RT, Silverson Machines, UK). The resultant w/o/w emulsion was magnetically stirred at room temperature overnight to evaporate the organic solvent. The particles were collected by centrifugation (Beckman J2-21, USA) for 15 min at 20.000 rpm and washed once with deionised water. The resultant suspension was freeze-dried with trehalose (5 % of total solid) (Virtis, Model ……, UK) to obtain a fine, dry powder of particles.

The protocol from where it all started...

(London, September 2006)

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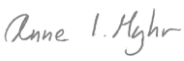
Anne Ingeborg Myhr	AIM	
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ACKNOWLEDGEMENTS

The work for this thesis was carried out at the Faculty of Biosciences, Fisheries and Economics (BFE), University of Tromsø (UIT), the Norwegian School of Veterinary Science (NVH) in Oslo and the School of Pharmacy (SOP), University of London in the period from 2006 to 2011. Financial support was provided by the Research Council of Norway (contract numbers 172508 and 183204), the University of Tromsø and *Tromsø forskningsstiftelse*.

First, I would like to acknowledge my two supervisors, Professor Jarl Bøgwald and Dr. Roy Dalmo for believing in me and providing me with the opportunity to take a PhD.

There are many people that have helped me along the way who I owe greatly for my achievements:

- Collaborators at SOP in London (where most of it started): Majella Lane, Jonathan Hadgraft, Liam McAuley, Gabbi, Paulo, Kaz. Even though it was a short stay, you made it highly memorable.
- Collaborators at NVH in Oslo: Hetron Munang'andu, Stephen Mutuloki, Cheng Xu, Inderjit Marjara and Øystein Evensen. Amr Gamil, Tove Hansen, Ane Sandtrø are acknowledged for the numerous hours and days spent to help us out with sampling *at Havbruksstasjonen i Tromsø* and Øyvind Haugland for helping me out with the QPCR assays. To all of you at BasAM; it has been a true pleasure to get to know you and thank you for making me feel at home. Øystein, thank you for including me in your research group and providing me with an office at NVH the last 2 years.
- Master students: Stine Mari Myren, Karina Sævareid and Jostein Grip. Your help and company in the lab have really been invaluable to me.
- To all colleagues in Tromsø and especially everyone at IMAB for the friendly atmosphere, helpfulness whenever needed and all the fun times both at work and in numerous social settings. A special thanks to Natasha Hynes for your friendship and all the help you have given me – it will never be forgotten.
- I would never have made this without my co-authors. The collaboration with Kåre Nolde Nielsen and Hetron have been especially appreciated; Kåre for introducing me to a different scientific area during the process of writing paper V and Hetron who gladly shared his office with me. Not only have you been the perfect office mate, but also contributed greatly to my development and thinking.
- And thanks also to Natasha, Tina Svingerud, my supervisors, Frode F. Fridell, Knut-Egil Løkling and Marianne Frøystad-Saugen for providing critical feed-back on this document.
- Invaluable technical assistance has been provided by Cristina Øie (isotope labeling), Merete Skar (PCS) and *Havbruksstasjonen i Tromsø* (tending of fish).
- To Professor Jorunn Jørgensen who supervised me through my MSc: Your inspiration and dedication to my scientific writing is still highly appreciated. Your good reputation will always be one step ahead of you.
- Mom, dad and my sisters for your moral support through all my studies. Grandpa Martin for your nice company and excellent Sunday dinners, as well as providing me with a place to stay during my final months in Tromsø. And most importantly to my little family; Hilde and Edvard who I hold so dear. Your love and endless patience and support are what made me finish.

Børge N. Fredriksen

Oslo, January 2012

LIST OF PAPERS

PAPER I

Early immune responses in Atlantic salmon (*Salmo salar* L) after immunization with PLGA nanoparticles loaded with a model antigen and β-glucan

B.N. Fredriksen, K. Sævareid, L. McAuley, M.E. Lane, J. Bøgwald, R.A. Dalmo.

Vaccine 2011 October; 29(46): 8338-8349

PAPER II

PLGA/PLA micro- and nanoparticle formulations serve as antigen depots and induce elevated humoral responses after immunization of Atlantic salmon (*Salmo salar* L)

B.N. Fredriksen and J. Grip.

Vaccine 2012 January; 30(3): 656-667

PAPER III

Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L)

H.M. Munang'andu, B. N. Fredriksen, S. Mutoloki, B. Brudeseth, T.Y. Kuo, I. S. Marjara, R.A. Dalmo and Ø. Evensen.

Manuscript submitted to *Vaccine* (December 2011). Accepted in revised form April 2012.

PAPER IV

Optimization of formulation variables to increase antigen entrapment in PLGA particles

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

Manuscript

PAPER V

Mapping uncertainties in the upstream: the case of PLGA nanoparticles in salmon vaccines*

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Nanoethics 2011 April; 5(1): 57–71

* Multidisciplinary work combining natural and social sciences (philosophy). The undersigned contributed mainly to p.59-64 and participated in discussions.

SUMMARY

Vaccines are regarded as the safest and most cost-effective strategy to prevent infectious diseases. For some diseases, vaccine improvements are required as protection levels are still inadequate. The key to solving this challenge might lie in the development of more efficacious vaccine delivery systems and adjuvants. Poly (lactide-*co*-glycolide) (PLGA) is a biodegradable polymer which has an extensive safety record in biological systems and possesses immunological adjuvant properties as injectable particles. In the present work, micro- and nanoparticles of PLGA and PLA were explored as a vaccine delivery system in Atlantic salmon (*Salmo salar*). The overall objectives were to investigate their adjuvant abilities in provoking innate and adaptive immune responses, forming antigen depots and inducing protective immunity in a challenge test with infectious pancreatic necrosis virus (IPNV).

Formulation parameters in preparation of polymeric particles were systematically optimized (paper IV) to achieve stable PLGA particle products containing co-entrapped model antigens and β-glucan (paper I and II), or virus particles of infectious pancreatic necrosis virus (IPNV) (paper III). Post immunization potency of nanoparticles (300-400 nm) was demonstrated by their ability to induce early innate responses (day 2, 4 and 8) at transcription levels equal to or higher than the oil-adjuvanted formulation (paper I). Temporal differences in expression levels of innate markers were observed, suggesting rapid systemic distribution of particles (paper I). By tracing of isotope labelled proteins, nanoparticles (< 1000 nm) were found to localize antigens in the head kidney while micro-sized (~ 8 µm) particles generally retained antigen at the injection site. Irrespective of size, particles made of polymers with high molecular weight (MW) generally had superior depot capabilities compared to their low MW counterparts (paper II). Adaptive immune responses to immunization were assessed by QPCR and ELISA. T cell markers were not differentially expressed at the selected early time points (paper I), but at day 60 and 75 antibody responses were found to be elevated (paper II and III). In a dose-response study, micro- but not nanoparticles were demonstrated to be equally potent compared to the oil-adjuvanted control group with regard to induction of antibody responses. Long-term antibody responses induced by particles were generally less robust and therefore declined towards the end of the experimental period (120 days), while responses induced by the oil-adjuvanted formulation progressively increased. Following immunization, antibody responses were not related to

polymer qualities or the ability of particles to depot or distribute antigens. Scoring of side effects demonstrated excellent safety profiles for the particle formulations (paper II and discussed in paper V). In paper III, vaccine efficacy was tested in a cohabitation challenge with IPN. Survival rates for the nanoparticle vaccinated groups were comparable to the non-vaccinated control fish and demonstrated that their ability to induce protection against IPN was inferior to the oil-adjuvanted vaccines. Virus re-isolation from head kidney and blood during the challenge period did however demonstrate some level of protection as the nanoparticle vaccinated groups were able to delay the IPNV infection.

In the presented studies, the principal adjuvant properties of PLGA particles in Atlantic salmon have been demonstrated to include their capacity to induce strong innate responses and provide antigen depots for long-term delivery of antigens. In addition, indication of particle presence in lymphoid organs was an interesting finding that could suggest a certain targeting effect to phagocytic cells. To achieve a better understanding of how PLGA particles may be used to direct immune responses in salmon, more detailed studies on particle qualities-cell interactions/responses are required.

OPPSUMMERING

Vaksiner er ansett som den sikreste og mest kostnadseffektive strategien for å forebygge smittsomme sykdommer. Mot noen sykdommer er beskyttelsen etter vaksinering fremdeles uttilstrekkelig og forbedringer av vaksinen nødvendig. Nøkkelen til å løse denne utfordringen kan ligge i utvikling av mer effektive vaksineleveringssystemer og adjuvanser. Poly (laktid-*co*-glykolid) (PLGA) er et nedbrytbart polymer som i en rekke studier har vist seg å være svært kompatibelt med bruk i biologiske systemer, samtidig som injiserbare partikler av PLGA innehar viktige immunologiske adjuvantegenskaper. I dette arbeidet har mikro-og nanopartikler av PLGA og PLA blitt utforsket som et alternativt vaksinekonsept til atlantisk laks (*Salmo salar*). Hensikten med arbeidet var blant annet å undersøke partiklenes evne til å framprovosere innate (medfødte) og adaptive immunresponser, fungere som antigendepoter og indusere beskyttende immunitet i en smittetest med infeksiøs pankreas nekrose virus (IPNV).

Ved en systematisk tilnærming ble formuleringsparametrene for produksjon av partikler optimalisert (artikkel IV) for å oppnå stabile PLGA partikler inneholdende modellantigener og β-glukan (artikkel I og II), eller viruspartikler av infeksiøs pankreas nekrose virus (IPNV) (artikkel III). Nanopartikler (300-400 nm) viste seg å være svært effektive i å indusere innate immunresponser (dag 2, 4 og 8 etter immunisering). På transkripsjonsnivå (mRNA) var responsene like eller høyere enn responsene etter immunisering med en olje-adjuvans (artikkel I). Temporale forskjeller i uttrykket av innate markører ble observert, noe som antydet rask systemisk distribusjon av partikler (artikkel I). Bruk av isotopmerkede proteiner viste at nanopartiklene (< 1000 nm) i stor grad bidro til å lokalisere antigenene til hodenyren, mens mikropartiklene (~ 8 μm) generelt holdt antigenet igjen på injeksjonsstedet. Uavhengig av partikkelstørrelse hadde partikler laget av polymerer med høy molekylvekt (MW) større kapasitet til deponere antigener i forhold til partikler laget av polymerer med lav MW (artikkel II). Adaptive immunresponser ble analysert ved bruk av QPCR og ELISA. QPCR-analyser viste at T-cellemarkørene ikke ble oppregulert i hodenyren eller milten de første dagene etter immunisering (artikkel I), men ved dag 60 og 75 var antistoffresponsene forhøyet sammenliknet med den negative kontrollgruppen (artikkel II og III). I et dose-respons studie ble mikro-, men ikke nanopartikler vist å være like potent i forhold til olje-adjuvansen med hensyn til induksjon av antistoffresponser. Antistoffresponsene etter immunisering med partikler var generelt mindre robuste i forhold til etter immunisering med en olje-basert adjuvans, hvor antistoffresponsen gradvis økte i løpet av den eksperimentelle perioden (120

dager). Antistoffresponser kunne generelt ikke er relateres til egenskaper ved polymeret eller partiklenes evne til å deponere eller distribuere antigener. Evaluering av bivirkninger i bukhulen etter immunisering med nano- og mikropartikler viste at disse formuleringene forårsaker svake eller ingen sammenvoksninger, men at melanindeponeringen er noe høyere i forhold til hos uvaksinert fisk (artikkel II og diskutert i artikkel V). I artikkel III ble vaksineeffekt testet i et kohabitantstudie med IPN. Overlevelsen for de gruppene som ble vaksinert med nanopartikler var sammenlignbare med den uvaksinerte kontrollgruppen og resultatene viste dessuten at partiklenes evne til å indusere beskyttelse mot IPN var dårligere enn beskyttelsen etter vaksinering med en inaktivert helvirusvaksine med olje-adjuvans. Re-isolering av virus fra hodenyren og blod i løpet av smitteperioden viste imidlertid at nanopartiklene hadde noen grad av beskyttelse ettersom disse gruppene var i stand til å utsette IPNV infeksjonen.

Studiene i denne avhandlingen viser at adjuvantegenskapene PLGA partikler har ved immunisering av atlantisk laks inkluderer deres evne til å indusere sterke innate responser og deponere antigener for langtidseksponering til immunceller. Indikasjon på tilstedeværelse av partikler i lymfoide organer er et interessant funn som antyder at partiklene kan benyttes til målrettet levering av antigener til fagocyttiske celler. For å oppnå en bedre forståelse av hvordan PLGA partikler kan benyttes til å dirigere immunresponsen hos laks, bør fremtidige studier fokusere på hvordan partiklenes egenskaper endrer partikkel-celleinteraksjonene og cellulært opptak.

PREFACE

During the last century, animal farming of both terrestrial and aquatic species has gone through a change from small businesses run by farmers and their families, to large scale production facilities with fewer owners, high production volumes and increased animal densities. The transition to the industrialized husbandry has constituted a challenge to farmers regarding preservation of the many aspects of animal welfare, specifically with regard to adaptation of prophylactic measures to limit the spread and outbreak of contagious diseases. Although the concept of vaccination originated more than 200 years ago, passive immunization and the use of antibiotics and chemotherapeutics has dominated disease prophylaxis and treatment in veterinary medicine until more recent decades. Acquired knowledge in virus propagation and adjuvant technology the last 50 years has albeit demonstrated that the basis for mass vaccination of farmed animals is present, cost-effective and a prerequisite for sustainable food production.

The Norwegian salmon farming industry was established during the 1960s and `70s. Salmonids (rainbow trout and Atlantic salmon) had favourable biological and behavioural conditions for farming, but while the technological challenges for husbandry were affordable, high production volumes remained absent due to outbreak of contagious bacterial diseases such as furunculosis (*Aeromonas salmonicida*) and vibriosis (*Vibrio salmonicida*[(*)] and *V. anguillarum*). Immersion and injection vaccines based on inactivated bacteria were initially tested on rainbow trout in 1977 and later in Atlantic salmon. Still, in the following years bacterial diseases became recurrent setbacks for the industry, an experience which is well reflected in the statistics over the use of antibiotics at that time (almost 50 metric tons in 1987 while fish production was merely 50 000 metric tons). It was not until an oil-based adjuvant was included in an injection vaccine during the late 1980s that the vaccines became potent enough to confer immunity against the aforementioned diseases. Today the annual Norwegian production volume of salmonids is close to 1 million metric tons (2010), a number that corresponds to about 250 million salmonids being vaccinated every year. The correct use of highly efficacious vaccines has reduced the use of antibiotics in salmonid farming by 99.8 %. The prescribed antibiotics the last years has mainly been to treat diseases in Atlantic cod, a species relatively new in the context of fish farming where vaccines still are in development. It is obvious that the use of vaccines during the last three decades have been important instruments for disease control and a condition for the continuous growth in the aquaculture industry.

In more recent years, intracellular pathogens have increasingly become a resource-intensive challenge. The oil-based adjuvant that so far has been a successful additive in vaccines seem to fell short in conferring protective immunity against some viral and intracellular bacterial diseases. Perhaps the best example in this context is the vaccine against infectious pancreatic necrosis (IPN). It was the first commercial viral vaccine for salmon to be introduced in the Norwegian marked in 1995. Even after years of research and optimization, the vaccines still only demonstrate sub-optimal protection and in 2009 alone, 223 outbreaks of IPN were recorded in Norway. With most of the bacterial diseases largely under control due to vaccination using oil-based adjuvants, the current challenge in vaccine development for the salmon aquaculture industry is to design and mass produce vaccine adjuvants and delivery systems that are able to mount robust immune responses and provide long-term herd immunity against intracellular pathogens. The recent characterization of emerging pathogens in salmonids, such as *piscine myocarditis virus* (PMCV) and *piscine reovirus* (PRV) (etiological agents for cardiomyopathy syndrome (CMS) and heart and skeletal muscle inflammation (HSMI), respectively) adds weight to this notion and emphasizes the need to search for novel vaccine concepts for future vaccinologists to be one step ahead of the pathogen.

"…may I not with perfect confidence congratulate my country and society at large on their beholding; in the mild form of the Cow Pox, an antidote that is capable of extirpating from the earth a disease which is every hour devouring its victims; a disease that has ever considered as the severest scourge of the human race!"

Final words by Edward Jenner (1749-1823) in
"*An inquiry into the causes and effects of the variolae vaccinae*".

INTRODUCTION

Even though vaccinology and immunology are different scientific disciplines they are found on the same branch in science and share a common goal; to understand how foreign substances affect the immune system. The current work combines the two fields in its presentation of a vaccine concept which still is considered novel in the context of fish. By mainly referring to experiments on higher vertebrates, the following introduction therefore aims to give some background on the vaccine delivery system and provide insight into how its characteristics may be used to affect and direct the immune responses in teleosts, with emphasis on salmonids.

AN INTRODUCTION TO FISH IMMUNOLOGY

Fish is a paraphyletic group of organisms consisting of almost 32 000 identified species[1], including about one hundred species of jawless hagfish and lampreys [1]. Fish have a unique evolutionary position as the most primitive of the vertebrates [2] and 400-500 million years ago it was the first animal phyla to possess both innate and adaptive immunity. Although fish have full representation of the fundamental components of the immune system, the level of sophistication appears to be somewhat different compared to mammals. The suggested trend is that the innate immunity is highly involved and richly diversified, while the adaptive immunity is less evolved and possibly less flexible [3]. As pointed out in two recent reviews by Whyte [4] and Magnadottir [5] the innate responses in fish may be considered vital due to both the late ontogenic appearance of the adaptive parameters in many species and the fact that the poikilotermic nature of fish may constrain the adaptive immune response.

The most apparent differences between the structure of the immune system of mammals and fish are the anatomical distribution of **lymphoid tissues** and the fact that fish lack lymph nodes and bone marrow. In teleosts the major lymphoid organs include the spleen, head kidney, thymus and the primitive gut/mucosa-associated lymphoid tissues (G/MALTs) as well as the recently described interbranchial lymphoid tissues [6-8]. The spleen and head kidney are known as filtering organs in the vascular system, removing effete blood cells and foreign agents, in addition to inducing and elaborating immune responses [9]. Due to their capacity to trap and present soluble and particulate antigens from the circulation, both these organs are

[1] Information retrieved from http://www.catalogueoflife.org/col/details/database/id/10 (12.12.2011)

considered lymph node analogues (secondary lymph organs) [10;11]. Furthermore, the head kidney is regarded a primary lymphoid organ because of its hematopoietic function and morphological similarities to the bone marrow found in higher vertebrates [4], and it is also the major site for B-cell development and antibody production [12]. Similar to other vertebrates the teleost thymus is considered the primary lymphoid organ for T-cell development [13;14] although T-cells have been suggested to assemble and aggregate in other tissues [8;15].

Like in mammals, **the innate (inherited) immune system** is divided into physical (epithelial/mucosal) and mechanical barriers, cellular components and humoral parameters (Table 1). In fish, the epithelial and mucosal linings are important portals of entry for pathogens as fish live in direct contact with their surroundings. Because of this, the skin, alimentary tract and gills contain numerous humoral defense parameters such as antimicrobial peptides (AMPs), immunoglobulins (Igs), complement factors, pathogen recognition receptors (PRRs) and cytokines [4;16-20] that exist either in soluble form or expressed as cellular receptors. **Natural antibodies** (immunoglobulins) in the form of IgM in teleosts, have been described in a number of fish species and are known to play a key role in the innate immune response [21-23]. A range of inflammation induced **acute phase proteins** (APPs) including C-reactive protein, serum amyloid P, lysozyme, transferrin and thrombin have been identified in teleosts, where hepatocytes are considered the primary source [24]. The APPs also comprise the **complement system**, which has a vital role in innate immunity by mediating phagocytosis, respiratory burst, chemotaxis and cell lysis [25] and on the adaptive immunity by augmenting B-cell proliferation [26]. All three complement pathways (alternative, lectin and classical) are well developed in fish and may result in the membrane attack complex (MAC), cell lysis and phagocytosis by opsonization. An important feature of the teleost complement system compared to what is found in mammals is the existence of several C3 subtypes with functional and structural diversity [27]. Furthermore, it has been suggested that cells of the monocyte-macrophage lineage do not express C3 [28].

Of the pattern recognition receptors (PRRs), the **Toll-like receptor (TLR)** family has been extensively studied in fish as these transmembrane proteins are regarded crucial in the detection of conserved structures on pathogens, collectively called **pattern associated molecular patterns**, or PAMPs [29]. The TLRs are clustered in two broad groups depending on the basis of their agonists; the TLRs responding to extracellular stimuli (e.g. TLR2 – peptidoglycan, TLR4 – lipopolysaccharides (LPS), TLR5 – flagellin) are located on the cell

surface, while the intracellular TLRs that recognize dsRNA and unmethylated CpGs include TLR3, 7, 8 and 9 and are located on the inner surface of endosomes [30]. The ability to distinguish among classes of pathogens makes the TLRs highly important in the orchestration of an appropriate acquired immune response. At least 16 different TLR types have been identified in fish, including 6 non-mammalian TLRs, and the agonist recognition induces cytokine expression similar to that observed in mammals [31-33]. Other widely used immune stimulating agonists in fish are **β-glucans** derived from bacteria, yeast and algae. The repetitive carbohydrate architecture found on glucans is known to associate with scavenger

Table 1. Summary of the main components of the innate and adaptive immune system in teleosts [4;24;34] .

Immune system division	**Immune system component**	**Effector mechanism**
Innate	Epithelial and mucosal linings; skin, alimentary tract and gills	First line of defense; physical barrier
	Humoral parameters	Antimicrobial peptides (AMPs), natural antibodies, the complement system and other acute phase proteins (C-reactive protein, serum amyloid P, lysozyme, transferrin and thrombin), associated cytokines, cellular receptors (PRRs).
	Cellular components	Granulocytes, non-specific cytotoxic cells (NCCs), monocytes/macrophages,
Adaptive	Cellular	Cytotoxic $CD8^+$T-lymphocytes (CTLs)
		$CD4^+$ T-helper lymphocytes (Th-cells)
		Associated cytokines
	Humoral	B-lymphocytes
		IgM, IgD, IgT and IgZ antibodies
		Associated cytokines
Lymphoid tissues	Primary	Head kidney (B lymphopoiesis) and thymus (T lymphopoiesis)
	Secondary	Head kidney and spleen
	Other	Gut- and mucosa-associated lymphoid tissues (GALTs/MALTs)
		Interbranchial lymphoid tissues (ILTs)

receptors, complement receptor 3, dectin-1 and TLR2/6 in mammals [35]. β-glucan receptors have been found in teleosts [36;37], but their receptor family affiliation remains to be elucidated.

The **cellular arm of the innate components** in teleosts includes cells that are morphological and functional equivalent to mammalian monocytes($B7^+$)/macrophages, granulocytes (neutrophils, eosinophils and basophils), thrombocytes and natural killer cells [4;6;38-40]. In addition, cells showing morphological similarities and expression of genes associated with dendritic cell (DC) function and antigen presentation have recently been described in zebrafish [41]. Of the innate cells, the defense primarily involves phagocytic (neutrophils and monocytes/macrophages) and nonspecific cytotoxic cells (NCCs). Phagocytosis followed by antigen presentation to adaptive immune cells, is central in mitigation and eradication of pathogens and is known to be a vital event that bridges the innate and the adaptive immune systems by certifying the development of a pathogen specific adaptive response. In mammals antigen presenting cells (APCs) are well described and include monocytes, macrophages, B-cells, classical dendritic cells (DCs) and plasmacytoid DCs [42]. However, in teleosts the macrophages are still regarded the most important professional cell in antigen processing and MHC II presentation, even though neutrophils and B-cells have been demonstrated to possess phagocytic activity [43;44].

Appearance of the thymus, the B- and T-lymphocytes and the RAG (recombination activation gene) enzymes are regarded as imperative for the evolution of the **adaptive immune system** as gene rearrangements offers an almost unlimited diversity in pathogen recognition. It is well established that all the basic features of the adaptive arm of the teleost immune system exist and that its initiation primarily relies on the ability of non-self discrimination and recognition performed by the innate components. **T-lymphocytes** in mammals are defined by expression of different cluster of differentiation (CD) glycoproteins and include effector and regulatory cells such as $CD8^+$ cytotoxic T-lymphocytes (CTL) and $CD4^+$ T-helper (Th) cell subsets known for their plasticity (Th1/Th2/Th9/Th17/induced regulatory T cells (iTreg)/T cell help for B cells (Tfh)) [45-48]. In some teleosts, T-cell related genes such as GATA-3, Foxp3, T-bet, TCR, CD3, CD28, CD4 and CD8 in addition to MHC class I and II genes have been identified [49-53] which suggest that the presence of $CD8^+$CTL and $CD4^+$ Th cells in fish are similar to those found in higher vertebrates. Fundamental assumptions have, however, been questioned with the recent finding of an distinctive immune system in Atlantic cod (*Gadus morhua*) where multiple MHC I genes and

unique composition of TLR families apparently compensate for the absence of MHC II and CD4 genes essential for a Th2 response [54]. Recently, a series of functional studies using monoclonal antibodies (mAbs) on teleost T-cells have demonstrated that allogeneically distinct leukocytes (assumed to be several types of APCs) were able to induce proliferation of $CD4^+$ lymphocytes followed by proliferation of $CD8^+$ lymphocytes [55] and that $CD8^+$ lymphocytes are the principal cell involved in cell-mediated cytotoxicity [56;57]. Furthermore, the basic characteristics (morphology, tissue distribution and gene expression) of $CD4^+$ and $CD8^+$ T lymphocytes were found to be similar in teleosts and mammals, although the low abundance of $CD8^+$ T cells in blood and distribution to the respiratory tissue may reveal a distinct dynamic in teleosts [8;58;59].

B-lymphocyte derived specific antibodies expressed either as receptors or secreted in plasma are key humoral parameters of the adaptive immune system. In general, the **antibody repertoire** of teleosts is regarded as more restricted compared to the mammalian counterpart [60]. Most importantly, the genetic organization of teleost Ig does not allow for class-switching, thus B-cells only express and secrete the IgM class, which is a trait similar to the B1 cells found in mammals. A fundamental characteristic of the teleost IgM is the tetrameric organization (pentameric organization in mammals) and the more loosely and flexible association of the monomers. Recent studies have also demonstrated the existence of other immunoglobulin isotypes, namely IgD [61;62], IgT [63] and IgZ [64]. As a response to vaccination, immunization or infection most teleosts mount strong antibody responses, however the lag period is known to be 4-10 weeks (species and temperature dependent) before significant levels can be detected, and antigen affinity and antibody heterogeneity are lower than those found in mammals [65]. In Atlantic cod and other species within the same family the humoral responses are described as weak and barely detectable after immunization, even though immunization with bacteria-derived antigens may induce protective immunity [66;67]. Immunological memory and initiation of secondary responses to previously encountered immunogens have been described as moderate or absent in teleosts [68], and the main reason for this could be ascribed to the lack of Ig class switching during second exposure, in concert with other factors such as slow metabolic rate and the absent of lymph nodes or germinal centers [34].

As a language between cells, **cytokines** (interleukins (ILs), interferons (IFNs) and chemokines) are known to bridge the innate and adaptive immune system and contribute to

tune and induce a pertinent response upon pathogen encounter. The lack of appropriate antibodies has so far limited research on teleost cytokines at the genetic (mRNA) level, although the biological activity of some central cytokines has been described [69-71]. However, their importance in the understanding of the teleost immune system is well reflected in the range of cytokines so far described at a genetic level, including features linked to inflammation (IL-1β, TNF-α, IL-2, IL-6 (acute phase), IL-8 (CXC chemokine), IL-17, IL-18), T-cell differentiation (IL-2, IL-4, IL-12), T-cell memory (IL-7, IL-15), viral persistence/survival factor for B and T cells (IL-21), antiviral defense (type I (IFNα(a)/β(b)) and II ((IFN-γ(g)/ IFN-γrel)) and anti-inflammation (IL-10), as reviewed in [40;72-76].

To sum up this section on fish immunology, the current understanding of the teleost immune system is that most of the qualities found in the immune system of higher vertebrates have an equivalent in one form or the other in teleosts. Even though there are species-to-species variations among teleosts, the basis for antigen recognition and initiation of the adaptive apparatus is tailored to enable effective eradication of invading pathogens, a quality which is fundamental for vaccinologists.

Vaccines and vaccination

A vaccine may be defined as "*a preparation of microorganisms or their antigenic components which can induce protective immunity against the appropriate pathogenic bacterium or virus but which does not itself cause disease*" [77] or simply as "*a dead or attenuated (non-pathogenic) form of the pathogen"* [78]. In addition to the immunogenic components, vaccines consist of an adjuvant/delivery system that aid in induction of innate and adaptive responses, and stabilizers/surfactants which contribute to the formulation/immunogens staying intact during storage and administration. In general, vaccines are further sorted in a number of sub-categories mainly based on the condition of the antigen. These include heat- or formalin inactivated whole microorganisms, antigen/immunogen sub-units (peptides, proteins, toxoids and its conjugates) and live/replicating/attenuated microorganisms, as well as plasmid DNA (pDNA) vaccines encoding immune inducing peptides/proteins of pathogenic origin.

ADJUVANTS AND VACCINE DELIVERY SYSTEMS

As the majority of non-living vaccines are relatively poor inducers of adaptive immunity, adjuvants (from the Latin word *adjuvare*, meaning "to help" or "aid") are essential components of most clinically used vaccines. In a broad sense, adjuvants comprise all substances that are able to accelerate, reinforce, improve or modify the effect of other agents (in vaccinology *other agents* refer to the antigen). Adjuvants are needed in a vaccine for various purposes, the foremost being to enhance the immunogenicity of highly purified or recombinant antigens and/or reduce the amount of antigen or the number of immunizations needed for protective immunity. Classification on chemistry and structure allocates vaccine adjuvants in two broad groups mainly based on their particulate and non-particulate nature [79]. Vaccine delivery systems are generally particulate adjuvants and comprise constructs such as emulsions (oil-in-water (e.g. MF59) and water-in-oil (Freund's adjuvant)), mineral salts (Al $(OH)_3$), liposomes, virus-like particles (VLPs), immune stimulating complexes (ISCOMs of saponin and lipid matrixes) and nano- and microparticles of chitosan, alginate and poly (lactide-co-glycolide) (PLGA) [80-86]. Non-particulate adjuvants are single compounds with intrinsic immunomodulatory and/or immunostimulant properties that generally benefit from association with a particulate adjuvant [79]. These include pathogen-derived products (e.g. lipopolysaccharides (LPS), unmethylated CpGs, myramyl dipeptide (MDP) and flagellin), carbohydrate products (e.g. β-glucan), synthetic products (double stranded RNA (poly I:C)) and endogenous immunostimulatory therapeutics such as cytokines [87]. For vaccines based on whole microorganisms, such as inactivated or live vaccines, a number of the non-particulate pathogen derived adjuvants are integrated as a natural part of the vaccine (e.g. flagellin found on bacteria or dsRNA in viruses).

ADJUVANT MECHANISM OF ACTION

Adjuvants were originally described as substances that when used in combination with a specific antigen would *"produce a more robust immune response than the antigen alone*" [88]. Even though the use of adjuvants has a long history, their exact mechanism of action is poorly understood as the effect of adjuvants mainly has been proven empirically. The general understanding is that adjuvants improve the immune response to vaccine antigens by; (1) increasing the immunogenicity of highly purified or recombinant antigens and thereby reduce the dose of antigen needed; (2) enhancing the magnitude, speed and duration of the immune response; (3) modulating antibody avidity, specificity, isotype or subclass distribution; (4)

stimulating CTL responses; and/or (5) generating antigen depots and/or pulsed antigen release [79;87;89]. In addition, the recognition of non-self [90] and tissue disruption caused by the vaccine matrix together provide exogenous and endogenous danger signals [91], respectively, that calls into action innate effectors able to prime T cells and thereby initiate appropriate adaptive responses. Moreover, administering antigens and adjuvants separately is known to result in considerably lower responses compared to co-injection, suggesting that the adjuvant effect is more synergistic than additive.

On a cellular level, vaccine recognition and subsequent initiation of appropriate responses are largely based on three signals. The most central signal is the antigen (signal 1), which provides the information required for development of specific immunity. Furthermore, co-stimulatory (signal 2) molecules via receptor-ligand interaction between APCs and T-cell antigens are required to avoid anergy and abortive responses. To activate APCs and orientate the Th response, an additional mandatory signal 0 is necessary. Signal 0 is mostly induced through the recognition of PAMPs including TLRs by PRRs. Depending on where the adjuvant acts on the recipient cell, adjuvants have been proposed to be categorized as type A, B or C [92]. Most of the recently developed receptor-specific immunomodulatory adjuvants such as TLRs ligands are categorized as type A, while the particulate adjuvants (i.e. vaccine delivery systems) are type B as they enhance antigen presentation to T cells by improving MHC conversion [79]. In this category, formulations such as liposomes, PLGA particles and oil emulsions are found. Type C adjuvants comprise adjuvants of endogenous origin that directly enhance signal 0 (e.g. cytokines). In brief, recognition of both the antigen and TLRs by APCs is required for optimal antigen processing and initiation of the innate, and subsequently the adaptive responses. Furthermore, several studies have demonstrated synergistic effects and increased vaccine potency by co-delivery of type A and type B adjuvants [93-96].

One of the first qualities to be described for adjuvants were their ability to depot antigens [97]. A depot ensures local retention of the antigen at the injection site and prolongs exposure of the immunogenic cargo to APCs. This quality is also assumed to be one of the central success factors of the oil emulsions used in salmon vaccines [98] and in addition to the powerful adjuvant properties it has contributed to make revaccination unnecessary. As mentioned previously, the immune response in fish may be hampered by a slow metabolic rate, and as a consequence this may influence the rate at which the immune response is mounted and make the antigen persistence necessary for an adaptive immune response to be

initiated before the antigen is cleared. Recent studies on the mechanism of action for the widely used alum and MF59 in humans have demonstrated that these adjuvants induce secretion of chemokines, which provides an immunocompetent micro-environment resulting in successive waves of infiltrating cell populations, with neutrophils being the first and most abundant, followed by inflammatory monocytes, eosinophils and DCs [99-101]. Similarly, in a study whereby salmon were injected intraperitoneally with an oil based vaccine, the early inflammatory responses were demonstrated to occur 1-2 weeks after vaccination and recognized by infiltration of neutrophils to the peritoneal cavity. During the next four weeks post immunization the number of neutrophils declined, while macrophages became more prominent. At even later time points (12-16 weeks) after vaccination, the number of lymphocytes increased [102]. In another study by the same authors, a steady decrease of the quantity of antigens was observed at the injection site from 3 to 12 months post vaccination [103]. Other studies have demonstrated that antigens accumulate in the head kidney and spleen [11;104], and durable persistence has been reported in the head kidney [11;104;105]. Together these studies demonstrate that antigen processing and clearance from oil based vaccines are consuming and possibly results in a sustained net transport of antigens to lymphoid organs. Drainage of antigens to lymph nodes has also been shown to be one of the adjuvant properties of the aforementioned MF59 and alum [99].

One of the long-term effects of vaccination of salmon with oil based formulations is the formation of classical immune granulomas where oil droplets and antigens are surrounded by macrophages intermixed with a few lymphocytes [103]. It has been speculated if granulomas serve as lymph node analogues as they provide a focal site for concurrent presence of innate and adaptive immune cells in a local cytokine micro-environment [98].

VACCINE EFFICACY AND SAFETY

The *in vivo* usability of commercially available vaccines for fish is primarily tested and documented on the basis of two main considerations. These include *efficacy*[2] (ability to reduce mortality and pathology, delay onset of mortality and induce lasting protection) in

[2] European Pharmacopeia 6.0 (01/2008:50207; "5.2.7 Evaluation of efficacy for veterinary vaccines and immunosera")

challenge tests and *safety*[3] (evaluation of adverse effects such as mortality, organ adhesion, reduction of growth, behavioural changes and melanization on organs and tissues) [104]. In the development of new adjuvants for use in veterinary medicine, safety and efficacy are key issues, but a trade-off between the two is often the case; an increased safety profile for highly purified antigens often results in decreased immunogenicity of the vaccine [106]. The difficulty in balancing the two is well reflected in the low number of delivery systems commercialized both in veterinary and human medicine. For human vaccines, a major unsolved challenge in adjuvant development is to achieve a potent adjuvant effect while avoiding reactogenicity or toxicity. Even though many vaccine delivery system are in the pipeline, MF59 (squalene) and alum ($Al(OH)_3$) are the only approved vaccine adjuvants in the majority of countries worldwide, where the latter still remain the standard [101]. In salmon vaccinology, substantial research has been devoted to finding alternatives to the mineral oil based adjuvant frequently used in injection vaccines. The reason for this has been ascribed to its highly reactogenic qualities causing adverse effects, where incidences of intra-abdominal lesions (organ adhesions), melanization in muscle, spinal deformities, reduced weight gain and long-term immunopathology (autoimmunity) has been reported [103;104;107-110] and the severity of the injections site reactions correlated to high expression of inflammatory markers [111]. Continued optimization of the vaccination regime and formulations may contribute to reduce the adverse effects typically seen for these vaccines [107;108;112]. However, many of the present generation of successful vaccines for salmonids are still based on oil adjuvanted delivery systems characterized by Th2-biased responses and high antibody titers [113] efficacious in preventing bacterial diseases [114;115], while efficacy against some intracellular pathogens remain inadequate.

In vaccine development, a key challenge is to understand the underlying differences between the immune responses induced by live replicating pathogens and the inactivated variants of the antigens used in vaccine formulations, especially in the context of vaccination against intracellular pathogens. In this regard, live attenuated and DNA vaccines have shown great prospective as alternative vaccines [116] as these vaccines rely on the natural invasiveness of the pathogen and the endogenous machinery of the host cells, respectively. They appear to offer significant potential for the induction of Th1-biased/CTL responses which have been one of the challenges faced with conventional oil based adjuvants. However,

[3] European Pharmacopeia 6.0 (01/2008:50206; "5.2.6 Evaluation of safety for veterinary vaccines and immunosera")

the safety of live attenuated and DNA vaccines for use in fish is more a concern than their efficacy due to the risk of reversion to virulence and e.g. the integration into chromosomal DNA, respectively [117;118]. Nevertheless, a DNA vaccine[4] against infectious haematopoietic necrosis virus (IHNV) and the use of *Arthrobacter davidanieli* as a live vaccine against *Renibacterium salmoninarum* and *Piscirickettsia salmonis* (causative agents for bacterial kidney disease (BKD) and salmonid rickettsial septicaemia (SRS), respectively)) have been approved for use on salmonids in the North American markets [21;119;120]. Much research has been devoted to bias the immune responses of inactivated vaccines towards induction of CTL effectors and furthermore identify safe alternatives that may contribute to reducing the use of oil-based adjuvants in salmonids and other teleosts, including a range of TLR ligands/immunostimulants [121-123] and novel carrier systems [124-128]. In the attempt to design the ideal vaccine (Table 2) balancing efficacy and safety, it remains to be seen if the novel adjuvants currently being explored will be implemented as a replacement or a complement to the oil adjuvanted vaccine delivery system so widely used in aquaculture.

ADMINISTRATION OF FISH VACCINES

Vaccination of fish in aquaculture is performed by immersion (dipping in a diluted vaccine solution), injection (preferably intraperitoneally, but intramuscular for DNA vaccines) or by oral administration through feed [129]. The great advantage with oral administration of vaccine antigens is the reduced fish handling (including transport and sedation), however this method falls short due to lack of control over the dosage each individual fish receives and that antigens are prone to degradation before reaching the immune sensitive areas of the gut [130]. For salmonids, immersion vaccination is mainly used during the early life-stages just after reaching an immunocompetent size (0.5-1 g), and it allows for mass vaccination at a stage when injection is made difficult due to the small fish size [131]. Although both immersion and injection vaccines at present are integrated as part of the production routines in salmon aquaculture, oil-adjuvanted injection vaccines administered to parr of salmonids (about 30-50 g) are recognized as the most efficacious route of administration for disease protection in larger production animals [129]. For high value species such as salmon and rainbow trout, injection vaccines are cost effective and represent a potent alternative as they allow the use of

[4] Information retrieved from http://www.ah.novartis.com/aqua/en/index.shtml (04.12.2011)

Table 2. Properties of an ideal vaccine [132].

- Life-long immunity
- Broad protection against all variants of an organism
- Prevent disease transmission
- Induce effective immunity rapidly
- Effective in all vaccinated subjects
- Require few (ideally one) immunization to induce protection
- Preferably not administered by injection
- Cheap, stable and safe
- Transmit maternal immunity to the fetus

adjuvants and administration of predictable antigen doses in each vaccinated animal [117]. However, manual injection of vaccines in fish is considered labour intensive (2500-3000 fish/vaccinator/hour) and the handling stressful for the animal. As a consequence, most modern injection vaccines are multivalent containing antigens from up to seven pathogens[5] in a single injection and more effective automated vaccination machines capable of handling 20 000 fish/hour are in the pipeline[6].

PLGA polymers and polymeric constructs

Because of their ease of manufacturing and desirable characteristics, biodegradable synthetic polymers such as PLGA have been the focus of extensive research for several decades. The American Food and Drug Administration (FDA) has approved the use of PLGA in human and veterinary medicine, and currently it is utilized in a range of biomedical products such as sutures [133], controlled pharmaceutical delivery matrices [134;135] and temporary orthopedic fixtures [136]. The use of PLGA as matrix for injectable microparticles was developed during the 1980s [127;137-141], and since then, research addressing the application of *micro*particles for delivery of therapeutic and prophylactic drugs, including antigens and immunomodulators has accelerated considerably. A recent trend in PLGA vaccinology has, however, been to construct particles in the *nano*meter size-range in an

[5] Information retrieved from www.pharmaq.no/products/ (06.11.2011).

[6] Information retrieved from http://www.maskon.no/pages/vaksine.html (30.11.2011)

attempt to *mimic* pathogens and improve vaccine delivery targeted for APCs with the objective of inducing Th1-polarized responses [142].

TECHNIQUES USED TO PREPARE PLGA PARTICLES

There are three commonly used techniques to prepare nano- and microparticles of PLGA; coacervation, spray-drying and the double emulsion solvent evaporation/extraction method [140;143-146]. All employ a similar first step, where antigen in an aqueous phase is emulsified in an organic solvent to yield a water-in-oil (W_1/O) dispersion [147]. In the further process, the coacervation method involves several stages of polymer desolvation and hardening to form the solid particles, while spray-drying atomizes the particles in a flow of drying air at slightly elevated temperature. The most frequently used technique for antigen entrapment and vaccine preparation is the *double emulsion solvent evaporation/extraction* method wherein poly (vinyl alcohol) (PVA) is the most employed stabilizing agent since it forms particles of relatively small size and uniform size distribution [148-153;153-156]. The reader is referred to Figure 2 for further details on the method of particle preparation, exemplified with formulation parameters used in the work presented in this thesis (papers I-IV). After final preparation, particles are lyophilized (freeze dried) to increase their shelf-life, resulting in a product as depicted by scanning electron microscopy (SEM) in Figure 1.

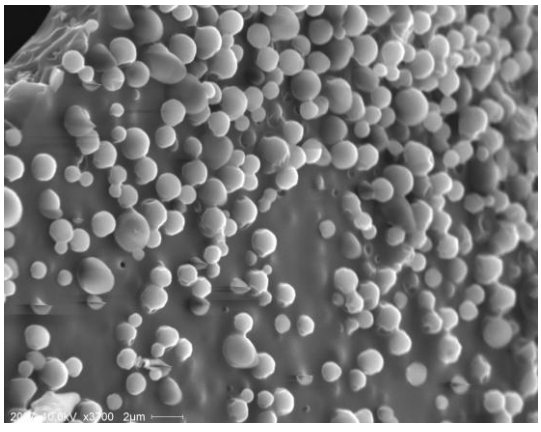

Figure 1. PLGA particles about 1-2 µm in diameter embedded on trehalose (dark background). Photo by Fredriksen 2008.

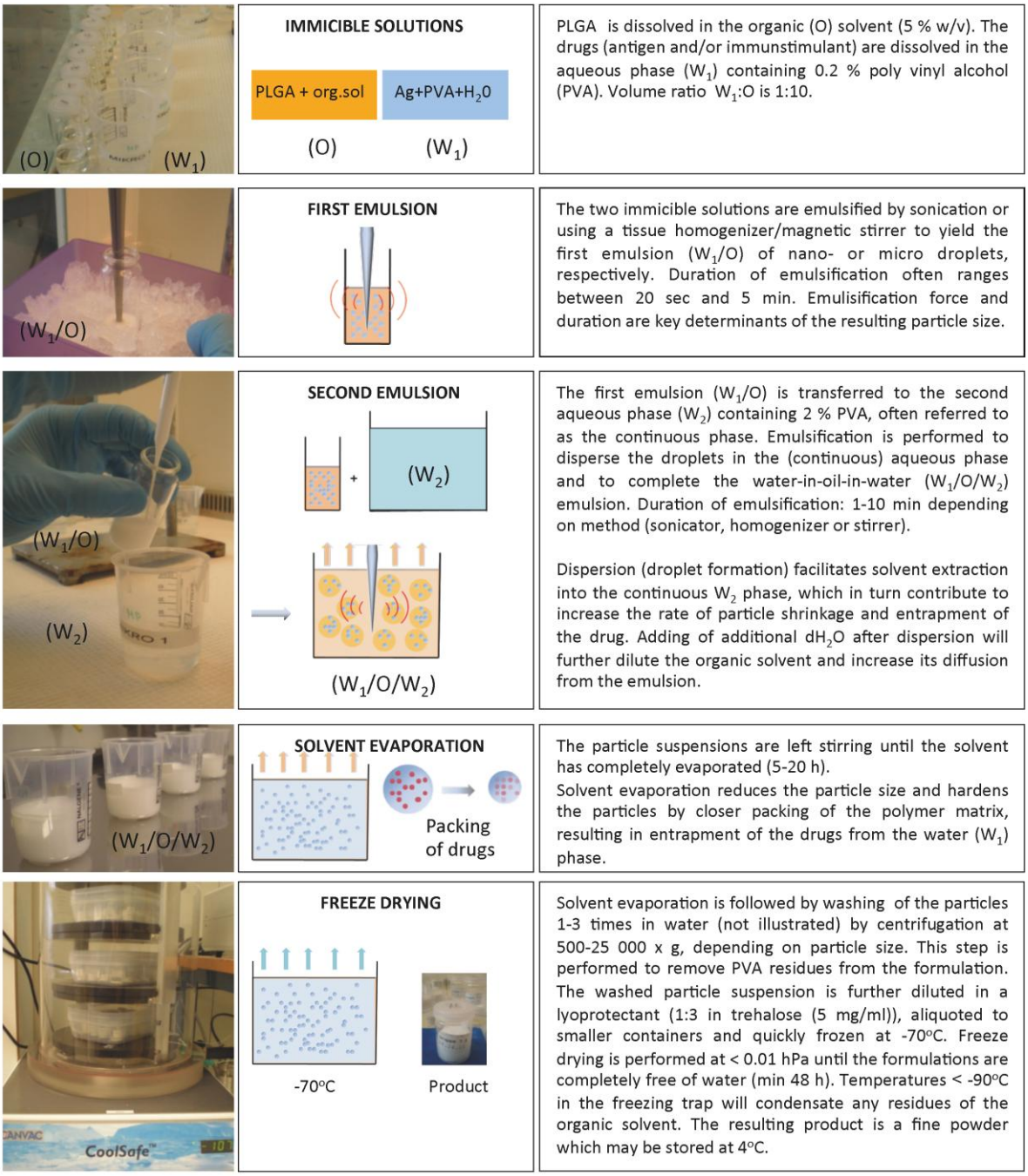

Figure 2. Overview of the *double emulsion solvent evaporation/extraction*-procedure used to prepare of PLGA and PLA particles. Pictures and illustrations by Fredriksen 2011.

QUALITIES OF PLGA POLYMERS IN VACCINES

Various qualities of the PLGA polymer may influence particle degradation and drug release and should therefore be considered in vaccine design. PLGA is a copolymer synthesized by random ring-opening copolymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid (Figure 3). During

polymerization, successive monomeric units are linked together by ester linkages, yielding a linear, amorphous aliphatic polyester product [157]. In general, PLGAs are thermoplastic synthetic polyesters with glass transition temperature (T_g) above 37^{o}C [158], hence they are glassy in nature and have a fairly rigid chain structure which gives them significant mechanical strength to be formulated as drug delivery devices [159;160]. For drug and antigen delivery, amorphous DL-PLGA (poly (DL-lactide-*co*-glycolide) acid) and DL-PLA (poly (DL-lactide) acid) has the preferred polymer stereochemistry because antigens are homogenously dispersed within these polymeric matrixes [161]. Furthermore, for preparation of vaccines mainly polymers with LA:GA monomer proportions ranging from 50:50 to 100:0 has been utilized [147]. In this context, the use of PGA (poly (glycolic) acid) is limited due to its lack of solubility in appropriate solvents for drug loading [162]. Of the two monomers lactic and glycolic acid, the former is more hydrophobic, thus is absorbs less water and subsequently degrades more slowly [137;138], which makes lactide-rich PLGA particles less prone to degradation. However, exceptions to this rule are the co-polymers with 50:50 ratio where the fastest degradation rate is observed due to the amorphous nature of the polymer [163]. Together with the great safety and tolerability profile seen for PLGA in biological systems [164], the possibility to vary LA:GA ratio in particle constructs is a key feature for its use as a vaccine delivery system as it allows for tailored design of vaccines with predictable antigen release kinetics [165]. Furthermore, surface potential (ζ potential) and functional groups are two important physiochemical characteristics of particles in vaccine design as they may influence cellular uptake and tissue distribution, which will be addressed later.

PLGA DEGRADATION

Polymer biomaterials can be divided into two main classes according to their lifetimes; namely biostable and biodegradable. The latter class includes PLGAs and are defined as polymers in which the degradation is at least mediated by a biological system [162]. PLGA is generally considered to degrade by non-enzymatic, autocatalytic cleavage of the ester linkages through spontaneous hydrolysis. This process is often referred to as a bulk erosion mechanism [166] and degradation takes place throughout the whole polymer matrix. During the first phase of hydrolysis the molecular weight of the polymer decreases significantly due to continuous cleavage and solubilization of low molecular weight fragments. At this stage no monomer products are formed and the construct still retains its original shape [167]. The middle phase (erosion or dissolution) of degradation is characterized by a rapid loss of mass

and formation of soluble oligomeric and D,L-lactic and glycolic acid monomers. In this phase the acidic microenvironment autocatalyze the further degradation. Complete polymer solubilization occurs when soluble oligomers are fragmented to soluble monomers [159]. Therefore, in addition to the LA content in the polymer, increased molecular weight contributes to extend the period (alter degradation behaviour) until complete polymer degradation occurs, an attribute that may be used in particle design to sustain antigen delivery [161]. PLGAs are known to be highly biocompatible and non-toxic [168], and after breakdown in the Kreb's cycle complete removal of the moieties (lactic and glycolic acids) from the body occurs through the respiratory route as carbon dioxide [169] or via excretion in the kidneys or the liver as carbon dioxide and water [159].

DRUG RELEASE FROM PLGA PARTICLES

PLGA particles either encapsulate drugs (in this context antigens and immunomodulators) or carry them on their surface through adsorption or covalent linkage [166]. Drug release from PLGA particles is most often assessed *in vitro* by incubation in a buffered aqueous solution, preferably at neutral pH. During incubation the encapsulated drugs are released into the surrounding buffer and successive sampling followed by analysis provides a picture of the release kinetics from the particles. For this purpose the bicinchoninic acid protein (BCA)/Smith assay [170] or high performance liquid chromatography (HPLC) are frequently

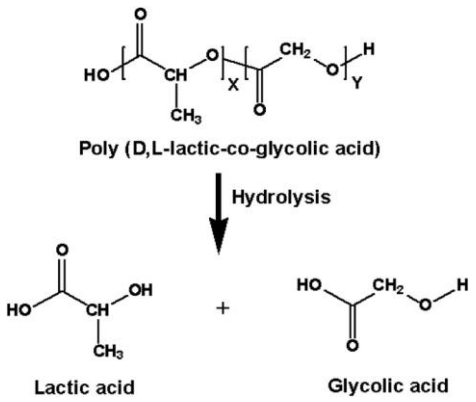

Figure 3. Chemical structure and biodegradation products of poly (lactic-*co*-glycolic) acid. The letters *x* and *y* denotes the ratio of lactic and glycolic acids in the polymer chain, respectively, hence a polymer with an equal number of the two monomers would be designated PLGA 50:50 (picture from [171]).

used methods. In the early phase of particle degradation, release of the entrapped drugs mainly occurs via diffusion in the polymer matrix, while release is mediated by a combination of diffusion and degradation of the polymer itself during the later stages [172]. Upon dispersion of particles in an aqueous solution, drugs absorbed to or near the surface are dissolved and diffuse out into the surrounding medium within a few hours or days, an event referred to as *burst* release [147;151;173]. Further release from the particles depends on particle porosity and hydrophilicity, as well as molecular interaction forces between polymer and drug [144;151;156]. In porous and hydrophilic particles there are less antigen-polymer affinity which facilitates water penetration into the particles while antigen diffuses out of the matrix [147]. Conversely, particles with dense cores, strong polymer-antigen interactions or a matrix of a hydrophobic polymer that restrict water uptake and subsequently pore formations, may have a *lag* phase with slow antigen release kinetics [147;151;156].

Theoretically, a reduced particle size would lead to increased surface area to volume ratio, resulting in a large area for hydrolysis to find place and therefore accelerated drug release. However, opposing results have been put forward suggesting that the degradation kinetics on the particle surface and in the core follow different patterns [151;174], emphasizing that the degradation of PLGA particles and preservation of drug stability (e.g. important epitopes on antigens) are complex events where release conditions such as temperature [174], residual surfactant (PVA) [153], additives/excipients (e.g. poly ethylene glycol) [175;176] and pH [177], in addition to particle morphology (size, porosity) should be considered when evaluating the polymer degradation and drug release. Drug/antigen stability during particle preparation and degradation/drug release is outside the scope of this text and the reader is referred to other literature for further reading on this subject [178].

CELLULAR UPTAKE OF PLGA PARTICLES

To achieve protective immunity from vaccination with inactivated vaccines it is required that the exogenous antigens are taken up, processed and presented by APCs. Uptake of antigens, immunomodulators and their carrier system by APCs is dependent on properties such as size, surface charge, shape and their hydrophobic/hydrophilic qualities, in addition to receptor interactions [179]. In comparison to emulsions, ISCOMs, TLR agonists and saponins, PLGA and liposome particles are considered inert depot forming carriers, unless they have a specific composition or carry immunostimulants [92;180].

The cellular uptake of nano- and microsized particles of PLGA has been well documented in macrophages and DCs [181-185] as well as epithelial cells [148;186] and vascular smooth muscle cells [187] in mammals. Depending on their size, particles may be incorporated in APCs via phagocytosis (0.5-10 μm), fluid phase (macro-) pinocytosis (0.5-5 μm) or clathrin-coated pits (< 200 nm) [188;189]. Efficient internalization of small particles is assumed to be attributed to the comparable size particle carriers have to the pathogens the immune system has evolved to combat [190]. The upper limit for particle phagocytosis by APCs is regarded to be in the range of 5-10 μm in diameter. Moreover, particles possessing diameters of 2-3 μm have been shown to exhibit maximal attachment and uptake [191] while 500 nm have been suggested as the lower cut-off size for efficient phagocytosis [192;193]. As the size of particles changes from nanometer to micrometer size within the range for cellular uptake, a drastic reduction in uptake of particles is observed [186;194-196]. A study by Desai *et al* [195] revealed a 2.5 and 6 fold greater uptake (based on weight) of 0.1 μm diameter particles compared to particles of 1 μm and 10 μm, respectively. Similarly, in terms of number, the uptake of 0.1 μm diameter particles was shown to be about 3 000 and 7 000 000 times greater than the 1 μm and 10 μm counterparts, respectively [195]. Furthermore, efficiency of nanoparticle uptake is known to be influenced by the incubation time and particle concentration [182] with a steady state of uptake being reached within 1-2 hours of incubation [195;197]. Comparing high (37^{o}C) and low (4^{o}C) incubation temperatures have shown that higher temperatures are favourable for particle uptake [195].

In addition to particle size, surface potential (often referred to as zeta (ζ) potential) is important for particle uptake. It is acknowledged that particles with primary amines at the surface generally undergo larger uptake compared to those having negatively charged sulfate, hydroxyl or carboxyl groups. Hence, positively charged (cationic) particles have a higher rate of cell uptake compared to negatively charged (anionic) or neutral formulations, while charged particles in general are more attractive for uptake compared to neutral particles [198-201] which may be attributed to the nonspecific electrostatic interaction between cells and carriers. Moreover, hydrophobic microparticles and lipophilic nanoparticles have been shown to be more susceptible to phagocytosis than their hydrophilic counterparts [202].

The *lag* phase in the degradation model for PLGA particles is considered to be one of the advantageous for their use as a vaccine delivery system. The slow degradation rate hinders early release of the immunogenic cargo before the particles are internalized in APCs, in addition to reducing the systemic distribution of entrapped molecules [171]. In mice, the site

of administration has been shown to significantly affect the type of cells that internalize particles. Particles of PLGA are mainly taken up by DCs following intradermal [183] or subcutaneous [203] administration, whereas macrophages have been found to be the predominant cells internalizing particles from the peritoneal cavity [183]. Figure 4 shows scanning electron microscopy photos taken during uptake of PLGA particles (diameter of about 1-2 µm) in *ex vivo* adherent cells (presumably macrophages) isolated from Atlantic salmon head kidney. The intracellular fate of particles after uptake in APCs is, however, still poorly understood and debated [204], but particle delivery has been demonstrated to result in MHC class I and II processing pathway of antigens, as summarized by Hamdy *et al* [171]. Nanoparticles have been shown to colocalize with early endosomes short time after uptake and it is generally acknowledged that the increasingly acidic endosomal compartments in APCs will accelerate PLGA degradation resulting in release of the encapsulated cargo, and furthermore, for efficient cross-presentation (MHC I pathway) to take place, either the encapsulated antigens *or* the PLGA particles have to enter the cytoplasm [205]. In a frequently cited study by Panyam *et al* [206] it was suggested that a reversal of the surface charge (from anionic to cationic) of particles in the acidic endo-lysosomal compartment causes the particles to interact with the endo-lysosomal membrane resulting in rapid escape of the particle into the cytosol [206]. By cytosol extraction, Shen *et al* [207] confirmed that antigen delivery via PLGA particles in fact increases the amount of exogenous antigens that escape endosomes and enter into the cytosol. However, other studies have demonstrated that particles may persist inside the early endosomes for up to 15 days suggesting that particle hydrolysis takes place in this compartment [208]. In a recent study by Schliehe *et al* [204], electron dense inorganic nanocrystals were encapsulated in PLGA microparticles to study their intracellular localization. Lysosomal storage of PLGA particles was found and the

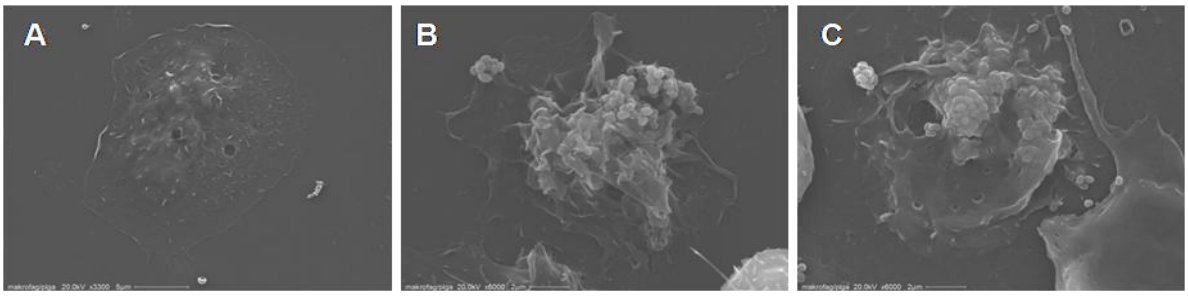

Figure 4. Uptake of PLGA microparticles (MPs) (~1 µm) in adherent *ex vivo* head kidney leukocytes from Atlantic salmon viewed by scanning electron microscopy (SEM, 20.0 kV, magnitude 6000x). Scale bar 2 µm. (A) Untreated adherent head kidney cell. (B) PLGA MPs outside/attached to adherent cell. (C) Internalized PLGA MPs in adherent cell. Photos by Fredriksen 2008.

authors concluded that cross-presentation only occurs after translocation of the protein, but not the particles, from the phagolysosomal compartment into the cytosol [204]. Irrespective of intracellular degradation and localization, comprehensive research point to the fact that particulate antigen delivery favours extended antigen presentation by APCs, which will be addressed in a later section.

ANTIGEN DEPOT AND BIODISTRIBUTION OF PLGA PARTICLES

For the purpose of antigen delivery, particle size and qualities such as surface charge may be important determinants to predict antigen location and kinetics. Due to their large size, microparticles of more than 5-10 μm are unable to relocate or be transported as intracellular cargo [209]. They will therefore form extracellular depots at the injection site from where they may slowly release their content. To the contrary, nanoparticles may be re-localized via the systemic circulation following administration, either as cargo in cells or by permeating biological barriers [210]. Once in the bloodstream, particles may interact with plasma proteins and blood components [198] and rapidly become sequestrated by the mononuclear phagocytic system. Intravenously injected particles have been shown to accumulate in the liver or spleen [175;211;212], whereas oral delivery has demonstrated distribution to liver, kidney, heart, brain, lungs and spleen [168]. After intramuscular administration, microparticles have been found in lymph node residing $CD11^+$ APCs for up to 120 days [208].

Grafting, conjugation or absorption of hydrophilic polymers, most notably poly (ethylene glycol) (PEG) is a surface functionalization that may be used to increase residence time in blood, reduce non-specific distribution or target cells/tissues with targeting ligands [213]. It has been demonstrated that the so-called *PEGylation* may drastically reduce uptake in the liver, whereas uptake in the spleen is increased [214]. Similarly, reduced uptake has also been proposed to come as a result of residual PVA giving higher hydrophilicity of the particle surface [153;215]. Hence, by altering surface properties it is possible to avoid hepatic metabolism and secretion, as well as increase the immunogenic load in lymphoid tissues.

PLGA PARTICLES AS VACCINE ADJUVANTS

The adjuvant properties of PLGA particles is quite complex as it acts on several levels, including some which have already been touched upon in previous sections. In general, the uptake of antigens and immunostimulants by APCs is known to be favored by delivery in a particulate form rather than soluble [216]. Particles can furthermore protect the antigen from premature proteolytic degradation [217] and may serve as antigen vehicles and depots both after injection and oral delivery [218;219]. In therapeutic and prophylactic immunology, a wide of range of immunostimulants and antigens have been encapsulated or absorbed to PLGA particles, including TLR agonists (e.g. LPS [220], MPLA [94;142], CpG [221-224] and poly (I:C) [223;225]) or other immunostimulants (e.g. β-glucan [93;226]) alone or in combination with antigens (see review by Hamdy *et al* [171]), whole viral particles (paper III, [227]), bacterial vaccines (particularly against tetanus [176;228-237], recombinant proteins [238] and plasmid DNA [208;239].

PLGA particles are known to increase the potency of a vaccine formulation [233;240]. By the use of particle delivery of antigens, improved cytosolic delivery of antigens have been shown to increase the access of exogenous antigen to the MHC class I loading pathway, stimulation IL-2 secretion by T cells at 1000- and 10-fold lower concentration compared to soluble antigens and antigen-coated latex beads, respectively [207]. The rapid and extended uptake of particles seen for professional APCs also indicates a certain targeting function. This property can be further enhanced by co-encapsulating TLR-ligands or other targeting molecules [241]. By incorporation DC-specific targeting antibodies on PEG-coated PLGA nanoparticles, Cruz *et al* [217] demonstrated that antigen dependent T cell responses could be induced at 10-100 fold lower concentrations compared to non-targeted nanoparticles. As demonstrated by Newman *et al* [242], PLGA particles may also contribute to provoke and enhance responses even against poor immunogens.

In vivo studies using ‘empty’ particles have suggested that particles alone can serve as complete adjuvant systems without the addition of TLR ligands. In a study by Sharp *et al* [243] DCs were primed with particles of different sizes (430 nm, 1 µm, 10 µm and 32 µm) to establish the relationship between particle size and enhancement of inflammation. It was concluded that uptake of particulate adjuvants is required for their ability to promote IL-1β secretion and that 1 µm sized particles was more potent, followed by 430 nm, 10 µm and 32 µm sized particles. Investigating TNF-α and IL-1β secretion in macrophages have shown similar distinct size-dependent responses [244] while adding of TLR ligands or antigens to the

formulations may further enhance innate responses by IL-6 and IL-12 production [142;245;246] and prolong as well as enhance antigen presentation [207;246].

In the search for efficient vaccines against tetanus (as summarized in e.g. [247]), a range of studies have investigated the ability of PLGA particles in generating humoral responses. Generally, these studies have demonstrated that PLGA particles may be more potent inducers of antibodies compared to alum [248], which is an adjuvant known to be a strong inducer of Th2 biased responses [79]. Additionally, combining the two has demonstrated a synergistic effect, seen as a more robust response both in terms of duration and titer levels [233;237]. Alum was found to promote increased attachment of particles on macrophage surfaces for a considerable period of time [249]. Designing PLGA particles with different release kinetics Kanchan *et al* [250] have suggested that slow and continuous release from polymer particles is critical in eliciting improved memory antibody responses from single point immunization [250]. Although conflicting data has been put forward [251], several studies have indicated that immune responses from micron-sized particles generally promotes humoral (Th2) responses while nanoparticles (<1000 nm) promote cellular (Th1) responses [238;252-254]. Katare *et al* [254] compared humoral responses after administration of very large particles (50-150 µm), microparticles optimal for phagocytosis (2-8 µm) and small particles (< 2 µm). It was found that particles in the size range of 2-8 µm exhibited remarkable improvement in the antibody response, especially compared to the very large particles. From another study by the same authors, microparticles (also 2-8 µm in size) were found to elicit antibody titers without being phagocytosed, but merely non-specifically attached to the surface of macrophages due to their size and hydrophobic nature [255], which have also been suggested by others [196]. These findings were related to up-regulation of MHC class II molecules and promotion of IL-4 secretion, indicative of a Th2-type response. Several other studies have demonstrated high antibody levels using large particles [234;236;237;256;257] based on hydrophobic polymers [237]. It has been proposed that the continuous presence of antigen (e.g. released from large particles) results in high concentration of antigen in the extracellular space near APCs with the outcome of direct loading of antigens to the MHC class II pathway [258;259]. Antibody responses have also been found to depend on antigen load in microparticles (µg antigen/mg polymer). Katare *et al* [254] compared the ability of microparticles carrying high (28.2 µg/mg) and low (1.3 µg/mg) antigen loading to induce antibody responses. Microparticles with high antigen loading/particle resulted in higher and more sustained antibody titers, and with reference to

other reports [259;260], it was suggested that immune responses to antigens of exogenous origin is dependent on the concentration of antigen load inside the individual APC [254].

In the aforementioned study by Kanchan *et al* [255] it was moreover demonstrated that nanoparticles (compared to microparticles) induced higher levels of IFN-γ in concert with MHC class I up-regulation, suggesting that their nano-size contribute to intracellular phagosome-to-cytosol delivery of antigens and a Th1-type immune response. Similar strong Th1 and CTL biased responses with the use of PLGA particles have been shown in several studies, however the responses were not consistently based on *nano*-delivery. Shen *et al* [207] demonstrated potent $CD8^+$ T cell responses (based on IL-2 secretion) and prolonged intracellular antigen presence when investigating cross-presentation after *in vitro* delivery of OVA in particles. Similarly, immunization with CpG ODN coated PLGA microparticles resulted in a pronounced IFN-γ secretion by splenic $CD8^+$ cells in mice, as reported by [261], while Lee *et al* [223] showed enhancement in MHC class I restricted presentation of exogenous OVA when co-encapsulated with CpG ODN or poly (I:C), in addition to antigen-specific CD4 and CD8 T cell proliferation. In a more comprehensive *in vivo* study by Heit *et al* [222], microspheres were loaded with a recombinant protein and CpG ODN. Results showed that these particles could trigger clonal expansion of antigen specific CD4 and CD8 T cells, and the potency was demonstrated by protective and therapeutic intervention. By co-delivery of MPLA and OVA, Hamdy *et al* [262] confirmed these findings and demonstrated markedly increased *in vitro* $CD8^+$ T cell proliferative responses (stimulation index above 3000) and 13-fold increase in the *in vivo* expansion of $CD4^+$ T cells. Furthermore, Schlosser *et al* [263] demonstrated that co-encapsulation, compared to administration in a mixed solution, may be superior in induction of protective immunity against viral infections. Together the studies presented in this section demonstrate that polymer-based particle delivery systems provide numerous ways to induce complementary humoral and cellular immune responses.

PREVIOUS WORK ON THE USE OF PLGA PARTICLES AS FISH VACCINES

Except for the papers presented in this thesis, there are to date only six research articles available on the NCBI (PubMed) database[7] describing the use of PLGA particles as a vaccine delivery system in fish. These studies have addressed the use of PLGA as antigen/pDNA carriers for oral and parenteral (intraperitoneal) vaccine delivery in Atlantic salmon [264], rainbow trout (*Oncorhyncus mykiss*) [265;266], Japanese flounder (*Paralichthys olivaceus*) [127;239] and Indian major carp, *Labeo rohita* (rohu) [267].

By oral delivery of encapsulated model antigens (human gamma globulin, HGG) to rainbow trout, O'Donnell *et al* [264] demonstrated that PLGA carriers results in higher antigen levels in serum compared to soluble (free) antigens. Furthermore, combining microparticles with 85:15 and 50:50 lactide:glycolide ratio, a pulsatile presence of HGG antigens in serum was observed at day 6 and week 5 post oral intubation. Using the same model antigen Lavelle *et al* [266] showed that antigens associated with microparticles (PLG 50:50) increased the retention time, slowed the passage of antigen and protected the antigen from proteolysis through the digestive tract of rainbow trout. In addition more antigens were detected in the bloodstream. Although the results indicated that the antigen was partially protected during delivery, the subsequent antibody responses were found to be similar to the fish given soluble antigen. In a more recent study on rainbow trout, Altun *et al* [265] encapsulated the bacteria *Lactococcus garvieae*, presumably resulting in a vaccine with bacterial fractions embedded in the PLGA matrix. After oral vaccination and subsequent boost vaccinations, relative percent survival (RPS) was 63% and 44% at challenge 30 and 60 days post vaccination, respectively. Following a boost vaccination at day 61 RPS values of 71% and 64% was achieved at day 90 and 120, respectively.

In a series of studies by Tian *et al* on Japanese flounder, PLGA nano- and microparticles was employed for oral administration of a plasmid (DNA) vaccine encoding the major capsid protein (MCP) from lymphocystis disease (LCD) virus [127;239]. Quantitative PCR and immunofluorescense analysis of tissues revealed that the MCP gene was present in gills, intestine, spleen, kidney, muscle, liver and heart 10 and 90 days post immunization with microparticles. Furthermore, a progressively increasing antibody production in sera was observed until 9 weeks post immunization, diminishing slowly

[7] Keywords: PLGA, fish, vaccine, vaccination, microparticles, nanoparticles. As a comparison, the keyword "PLGA" alone yielded 5043 search results, while "PLGA microspheres" and "PLGA nanoparticles" yielded 1595 and 1140 search results, respectively. Information retrieved 05.12.2011

towards week 25 where MCP specific antibodies were no longer significantly different from the control [239]. In another study performed by the same researchers, oral vaccination with PLGA nanoparticles loaded with pDNA was followed by intramuscular challenge with LCDV. This study demonstrated that vaccination with PLGA/pDNA nanoparticles induced significant innate (e.g. lysozyme and respiratory burst) and adaptive (specific antibodies) responses in blood compared to the pDNA or the PLGA nanoparticles alone. More importantly, the vaccine contributed to drastically reduce the occurrence of nodules during disease progression and the immune parameters investigated could be correlated to the protection against the disease [127].

Instead of oral delivery, Behera *et al* [267] administered a PLGA microparticle split vaccine carrying the outer membrane protein (OMP) of *Aeromonas hydrophilia* by the intraperitoneal route. In accordance with the work by Tian and Yu [268] non-specific and adaptive (specific antibodies) immune parameters in blood were found to be significantly higher in PLGA immunized Indian major carp compared to a Freund's adjuvanted control at day 21 and 42 post immunization [267].

OBJECTIVES OF STUDY

The last two decades, extensive studies have been performed involving PLGA/PLA nano- and microparticles as adjuvants in delivery of immunogenic agents. The current study on the use of this antigen delivery system in Atlantic salmon was motivated by the advantageous prospects polymeric particle have demonstrated as single shot multiple dose vaccines and their ability to induce immune responses against intracellular pathogens. This study aimed to characterize some of the adjuvant properties PLGA/PLA nano- and microparticles may have in Atlantic salmon.

The specific objectives were to:

- Develop/establish a protocol on how to prepare and characterize nano- and micro sized PLGA and/or PLA particles for research purposes.

- Explore innate and adaptive immune responses to polymeric particles in Atlantic salmon.

- Investigate how particle properties such as size and molecular composition contribute to the antigen retention and biodistribution in Atlantic salmon.

- Evaluate vaccine efficacy of PLGA nanoparticles in Atlantic salmon after vaccination against and challenge with an intracellular pathogen.

ABSTRACT OF PAPERS

Paper I

Early immune responses in Atlantic salmon (*Salmo salar* L.) after immunization with PLGA nanoparticles loaded with a model antigen and β-glucan

Polymeric nanoparticles (NPs) of poly (lactic-co-glycolic) acid (PLGA) possess adjuvant properties. To date, there are few studies exploring their application as antigen carriers for vaccination of fish. This study presents a preclinical assessment of the early innate and adaptive immune responses in Atlantic salmon following immunization with PLGA NPs. A model antigen (TNP-LPH) and an immunostimulant (β-glucan) were entrapped in NPs of 300-400 nm either alone or in combination. Both the antigen and the β-glucan were efficiently entrapped (>50%) in particles and an antigen release study indicated particle stability up to 50 days at 8°C. Spleen and head kidney were analyzed for pro-inflammatory markers (TNF-α, IL-1β, IL-8, C3a) and T cell cytokines, effector molecules and transcription factors (IFN-γ, T-bet, GATA-3, granzyme A, IL-10, Foxp3) at mRNA transcription levels 2, 4 and 8 days post i.p. immunization. NPs alone were able to moderately up-regulate pro-inflammatory immune responses. Addition of immunogenic cargo, either an antigen or β-glucan generally increased the gene expression of pro-inflammatory markers, while administering both resulted in the highest gene expression. These findings were also reflected by concurrently increased levels of IL-10. Comparing the treatment groups injected with antigen and β-glucan co-administered either in NPs or FCA demonstrated that the magnitude of the acute pro-inflammatory responses was equal between the treatments or highest in the NP injected group. Although elevated expression of granzyme A in the NP injected groups (carrying antigen and/or β-glucan) was observed, PLGA NPs were unable to induce T cell differentiation on mRNA gene expression levels, as increased levels of the indicating cytokines and transcriptions factors failed to occur. In conclusion, this study demonstrates that PLGA NPs have potential as an adjuvant in salmon vaccines as they enhance the early pro-inflammatory responses to immunization.

Paper II

PLGA/PLA micro- and nanoparticle formulations serve as antigen depots and induce elevated humoral responses after immunization of Atlantic salmon (*Salmo salar* L.).

Novel vaccine delivery systems are highly needed to improve the salmon aquaculture industry. Although particles of biocompatible polymers such as poly(lactic-co-glycolic acid) (PLGA) have long been considered promising candidates for delivery of immunogenic compounds, few studies have addressed their use as vaccine carriers in Atlantic salmon (*Salmo salar* L.). Investigating their ability to retain/depot antigen and induce time and dosage dependent adaptive humoral responses to immunization, we here present a basic study of the adjuvantic properties PLGA and PLA particles may have in salmon vaccines. A model antigen (human gamma globulin, HGG) was co-encapsulated with β-glucan in nanoparticles (<1000 nm) and microparticles (~8 μm) of different chemical compositions. Atlantic salmon were immunized with (a) PLGA or PLA particle entrapped antigen (12 different treatment groups), (b) antigen and β-glucan in PBS, (c) an oil-based formulation or (d) nanoparticles (NPs) or microparticles (MPs) combined with the oil-adjuvanted formulation. ELISA analysis showed that NPs and MPs were capable of inducing elevated antibody responses at day 60 and 75 post immunization, but the antibody levels were reduced at day 90 and 120. In contrast, oil-based formulations, either alone or in combination with NPs or MPs resulted in strong antibody responses at all sampling time points. Comparable dosage dependent increase in antibody responses was observed when administering antigen with β-glucan either in PBS, entrapped in NPs or MPs, or in an oil-adjuvanted formulation. However, as the antigen doses were increased, MPs and the oil-based formulation gave the strongest responses. Antigen presence in the blood, organ package/injection site, kidney, carcass and the whole body was quantified by radiotracing of I^{125}-labelled HGG at day 7 and 36 post immunization. At both sampling time points, the highest radioactivity levels were measured from the whole-body and organ package/injection site in groups injected with MPs and oil-based formulations, indicating that these formulations resulted in superior antigen retention. Interestingly, NPs were found to accumulate in the kidney, a result that corroborated with in vitro uptake of NPs in a DC/Mφ-like cell line from Atlantic salmon.

Paper III

Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (*Salmo salar* L)

Two phenotypes of infectious pancreatic necrosis virus (IPNV) made by reverse genetics on the backbone of the Norwegian Sp strain NVI-015 (GenBank AY379740) encoding the virulent ($T_{217}A_{221}$) and avirulent ($P_{217}T_{221}$) motifs were used to prepare inactivated whole virus (IWV), nanoparticle vaccines with whole virus, *Escherichia coli* subunit encoding truncated VP2-TA and VP2-PT, VP2-TA and VP2-PT fusion antigens with putative translocating domains of *Pseudomonas aeruginosa* exotoxin, and plasmid DNA encoding Segment A of the TA strain. In general, Kaplan Meyer's survival plots reflecting the post challenge survival percentage (PCSP) for the different vaccines showed that inactivated whole viral (IWV) vaccines conferred highest PCSP (PCSP=42-53) while nanoparticle, sub-unit recombinant vaccines and the DNA vaccine fell short of the IWV when tested in Atlantic salmon (*Salmo salar* L) postsmolts challenged with the highly virulent Sp strain NVI-015 (TA phenotype) of IPNV. Antibody levels induced by these vaccines did not show phenotypic differences between the virulent and avirulent motifs for vaccines made with the same antigen dose and delivery system after 8 weeks of immune induction. Comparison of vaccine efficacy based on ability to prevent establishment of infection showed that fish vaccinated with less potent vaccines tend to get infected much earlier and yield to higher infection rates than fish vaccinated with highly potent vaccines after challenge. Ability of the virulent and avirulent motifs to limit the establishment of viral infection showed equal protection for vaccine phenotypes made of the same antigen dose and delivery systems. Prevention of tissue damage linked to viral infection was eminent in the more potent vaccines than the less protective ones. Hence, there still remains the challenge of developing highly efficacious vaccines with the ability to eliminate the post challenge carrier state in IPNV vaccinology.

Paper IV

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) vaccines when the antigen is of short supply. This study presents a systematic approach in the testing of formulation variables during PLGA particle preparation. The objective was to optimize/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 (I^{125}). 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 V

Mapping uncertainties in the upstream: The case of PLGA nanoparticles in salmon vaccines

The diversity of nanotechnologies and of the governance challenges that their applications raise calls for exploration and learning across different cases. We present an Upstream Oversight Assessment (UOA) of expected benefits and potential harms of nanoparticles made of a synthetic polymer (PLGA) to improve vaccines for farmed salmon. Suggested by Jennifer Kuzma and colleagues, an UOA may help identify and prioritise research needs, and it may support evaluations of the adequacy of relevant existing regulatory frameworks. In this work, the UOA approach is modified and supported with elements from the uncertainty analysis framework developed by Warren Walker and colleagues. Empirically, we draw on relevant available published literature and insights generated in an ongoing nanoparticle salmon vaccine project, in which one of the authors participates. Nanotechnologies have not previously been encountered in the regulatory context of fish vaccines, which in part raises unique challenges due to prospective large scale vaccine use in semi-open aquatic systems. Strengthened through cooperation between ELSA and technology researchers we found the UOA useful for an early mapping of benefits and concerns, and for identifying areas in need of further research prior to a nanoparticle based salmon vaccine is developed and taken into use. We consider our approach to represent one among several complementing initiatives that seek to contribute to early stage evaluations of possible negative side effects, broadly conceived, in order to facilitate a more robust nanotechnology development.

RESULTS AND GENERAL DISCUSSION

At the time this project was started, very little was known about the performance of PLGA particle vaccines in fish. Because of this, several fundamental questions regarding their adjuvant properties were sought answered, including their ability to be distributed to lymphoid organs and retain antigens, to induce innate and adaptive responses as well as safety and efficacy of the vaccine concept after immunization and challenge, respectively. As results have been discussed in detail in the included papers, the following text will mainly make a summary of the main findings and address matters of a more general significance.

Establishment of a protocol for preparation of PLGA particles

Of the three most utilized protocols to prepare PLGA particles [140;143-146], the double emulsion solvent evaporation (water-in-oil-in-water) method was chosen as it is a well established and well described method in scientific literature with respect to antigen and immunostimulant encapsulation. In design of vaccines for research purposes, the focus areas were to optimize/maximize entrapment efficiency of antigens and produce stable particles within predictable size ranges (~ 200-500 nm, 1-2 μm and 5-15 μm). During the establishment of the emulsification procedure in our laboratory, it was realized that some of the process variables described, and often cited in literature did not agree with the results obtained in our studies. By a systematic approach, process variables including (1) choice of organic solvent; (2) PVA concentration in the internal and external water phase; and (3) volume ratios were investigated with the sole aim to increase antigen entrapment in the particles. A general description of the methodology is cited in Figure 2, while results from the current work have been presented and discussed in paper IV. Key findings showed that the entrapment efficiency is highly dependent on PVA concentrations and that satisfactory production yields (> 70%) could be obtained using chloroform. More importantly, the observations presented in paper IV were implemented in the preparation protocols for the formulations used in paper I-III, where model antigens (TNP-LPH and OVA) and whole virus particles (IPNV) were successfully encapsulated. In paper I and II, antigen release studies were also included to determine particle stability. Although particles were made of PLGA and PLA with different chemical qualities (MW and LA:GA ratios) and different size ranges (<1000 nm or ~8 μm), it was demonstrated that particles generally were highly stable for up to 120 days irrespective of size and chemical properties (paper II). However, entrapment efficiency and loading of antigens and β-glucan were consistently lower in microparticles compared to nanoparticles (paper II),

but incorporation of β-glucan did not alter the entrapment or release of antigen notably (paper I). In general, the released antigen mainly came as a result of a *burst* release in both studies, while further incubation revealed an extended *lag* phase that did not accelerate considerably at the selected temperature (8^{o}C) and during the incubation time in the current studies. There were, however, considerable release kinetic differences between micro- and nanoparticles (paper II), probably due to release of surface bound antigens [147;151;173], particle associated PVA [153] and/or particle porosity [147], where the latter seemed plausible as high burst release may be coupled to low entrapment efficiency. Moreover, it is likely that antigens have escaped the PLGA matrix during solvent evaporation and particle hardening at preparation, thereby resulting in the lowered entrapment efficiency generally obtained in the microparticle formulations.

Temperature has been demonstrated to be an important factor both for cellular uptake of particles [195] and *in vitro* release [174] of antigens. Further studies on the stability of PLGA particles designed for salmon should therefore include particle incubation at higher temperatures (37^{o}C) to accelerate antigen release. This will stress test the formulations and provide stability results that are comparable to other studies where murine animal models have been simulated.

Safety profile for PLGA nano- and microparticles

As summarized in paper V (Table 1) only a few publications exist on the use of PLGA particles in fish and none of them have addressed safety aspects. To the author's knowledge, paper II is therefore the first to present safety data on the use of PLGA nano- and microparticles in fish. The *Speilberg-scale* [104] is well established for evaluating macroscopic side-effects in Atlantic salmon by scoring organ and tissue adhesions. Since nano- and microparticle formulations may display different retention characteristics at the injection site, it was expected that the various preparations could induce different side effect profiles. Thus, it was decided to score also melanin deposition and vaccine residues on a separate scale [269]. High incidence of particle residues has been found in the peritoneum and the incidence of adhesions shown to be linearly related to the particle size [210]. Kohane *et al* [210] also reported that nanoparticles resulted in fewer adhesions due to rapid clearance from the peritoneum, while high MW polymers induced more adhesion than low MW polymers did - irrespective of polymeric residues in the peritoneum. Immunization (paper II) was performed with length adjusted reusable medical needles with metal hubs to ensure vaccine

delivery in the peritoneum avoiding injection into surrounding tissues/organs. Furthermore, particles were formulated under sterile conditions, but no additional sterilization was carried out after the final step of freeze-drying prior to injection, as was reported by Kohane *et al* [210]. Nevertheless, our results demonstrated that PLGA and PLA nano- and microparticles in general caused fewer adhesions compared to the oil-emulsion, with scores under 1.0 for all groups. According to the *Speilberg-scale* a score of 1 is given when "very slight adhesions" are seen focused around the injection site, while 2 indicates "minor adhesions" where adhesions to the abdominal wall can be observed [104]. The side effects observed for the PLGA or PLA formulations in paper II could not be related to the MW of the polymer, as reported by others [210], but micro-sized particles generally caused more incidents of melanin in the peritoneal cavity possibly due to prolonged presence of particles. Even though this is indicative of an irritant effect [270], the findings presented in paper II suggest an excellent safety record for the use of PLGA and PLA particles in Atlantic salmon, which is in accordance with safety studies on the use of PLGA particles reported in other animal models [168]. However, in paper III and in an unpublished vaccine study where PLGA nanoparticles were used to vaccinate Atlantic cod against furunculosis (*Aeromonas salmonicida*), severe side effect were observed (but not systematically documented), as pointed out in paper V. From these observations as well as in accordance with the European Pharmacopeia for veterinary vaccines, it is obvious that the safety of PLGA vaccines should be evaluated both on the basis of antigen (origin and dose) and the species for which its use is intended.

PLGA particles induce inflammatory responses in Atlantic salmon

A cardinal adjuvant property is the ability to initiate innate immune responses. Although PLGA particles are known to be highly biocompatible, their potency as vaccine delivery systems has been demonstrated in a range of previous studies (as reviewed in e.g. [135;251]). In paper I, expression of the pro-inflammatory cytokines TNF-α and IL-1β, the chemokine IL-8 and complement factor C3 was quantified on mRNA transcript levels *in vivo* after immunization with different combinations of antigen (TNP-LPH) and β-glucan either encapsulated or in soluble form. Freunds Complete Adjuvant (FCA) was used as a positive control because it is known to cause inflammation [271]. PLGA nanoparticles were generally able to induce pro-inflammatory responses equal to or higher than FCA. Additionally, co-entrapment of antigens and β-glucan gave indications of an additive effect compared to injecting the substances separately (paper I), a finding that is in accordance with others

[222;261;263]. An interesting temporal difference was observed, with TNF-α expression in the spleen occurring earlier in the nanoparticle group (NP/antigen/β-glucan) compared to the oil-adjuvanted control group. Due to their small size and the fact that particles were injected suspended in PBS, a rapid systemic distribution of the particles should not be ruled out [210]. Coupled to the high levels of IL-1β and IL-8, it was speculated whether the early splenic expression of TNF-α reflected the presence of nanoparticles (paper I) as particulate antigens have been found to accumulate in the spleen in teleosts [10]. TNF-α is an important activator of macrophages in salmonids, resulting in increased phagocytosis and chemotaxis, as well as induction of IL-1β and IL-8 [4]. As nanoparticles failed to up-regulate genes related to adaptive immunity (discussed in the next section), but induced strong innate responses, an interesting approach would be to explore macrophage activity e.g. by IL-12 and MHC class I and II expression in future studies. Generally, incorporation of β-glucan in the PLGA particles indicated a certain ability of this immunomodulator to increase the potency of the formulation, as seen by up-regulation of all the innate markers. Together with the efficient entrapment of β-glucan in PLGA nanoparticles our results suggest that β-glucan should be further explored in the context of vaccine delivery using PLGA.

Adaptive responses to PLGA particles

Adaptive responses were investigated at early (day 2, 4 and 8) and late (day 60, 75, 90, 120) time points after immunization by Q-PCR analysis of central T cell genes (paper I) and ELISA analysis for detection of antigen-specific antibodies (paper II), respectively. In paper I, expression analysis of $CD4^+$ T cell genes (Th1 (IFN-γ and T-bet), Th2 (GATA-3), Treg (IL-10 and Foxp3) and (CTL (granzyme A)) were investigated. Based on the findings reported by others [205;207;222;223;238;248;255;261-263;272], it was hypothesized that nanoparticle formulations could increase endosomal escape of antigens and induce CTL responses (increased expression levels of granzyme A mRNA) and Th1-like responses detectable as increased IFN- γ and T-bet expression. However, none of the selected Th1 and Th2 genes were differentially expressed and only elevated levels of IL-10 and granzyme A were found. Taken together, the expression levels for these genes failed to reject the null hypothesis. The reason for the non-responsiveness obtained for the adaptive markers were not known, but generally it may be questioned whether the immunological load constituted by the vaccines was too low to induce such responses, whether sampling was performed too early after immunization, if the study design in terms of sampling size were too few for such responses

to be detected by QPCR or most importantly, if the formulation in itself would be able to induce such responses irrespective of the former points. As sequences for the transcription factors (GATA-3 [51], Foxp3 [53] and T-bet (registered on NCBI)) in salmon have been made available quite recently, no solid data has been presented on their regulation after immunization of fish. By gene analysis twelve weeks after vaccination, Mutuloki *et al* [111] reported of high expression of inflammatory markers and IL-17 (a potent mediator in delayed-type reactions) in correlation to side effects, but genes reflective of Th1 and Th2 responses were not differentially expressed. In a study by Haugland *et al* [273], T cell receptor genes were found to comprise a higher proportion of the up-regulated genes at day 19 compared to analysis performed at earlier time points (day 2 and 8). For future studies, later sampling time points as well as larger sample sizes should be included to rule out the possibility of the non-responsiveness being a question of inadequate study design.

In paper II, nano- (< 1000 nm) and microparticles (~ 8 μm) made of polymers with different qualities (MW and LA:GA) were investigated for their ability to induce specific antibody responses. Coupled to a study on antigen retention, the work aimed to examine the potency, magnitude and duration of the antibody responses in comparison to a group immunized with antigen emulsified in FCA, which is a combination known to be a strong inducer of humoral responses. In the dose-response study, potency and magnitude of the antibody production was demonstrated to be significantly higher in groups immunized with PLA-microparticles or FCA at doses of 100 μg antigen per injection (Fig. 3 in Paper II), which is in accordance with other studies comparing particle size and antibody responses [254;255]. Furthermore, antibody responses were found to be at their highest 60-75 days post immunization in groups injected with particle formulations suspended in PBS, while they decreased towards day 120. To the contrary, groups immunized with FCA alone or carrying nanoparticles or microparticles showed increased antibody responses towards day 120, when they were at their highest. These findings underline the superior ability of oil-adjuvanted vaccines to induce strong humoral responses in fish [104]. In mice, PLGA and PLA particles are able to induce strong antibody responses based on their size [254;255] and hydrophobic nature [237], and it has been demonstrated that combining nano- and microparticles may further enhance the responses [237]. Since a broad range of formulations were used in paper II and none of them were able to induce durable/robust antibody responses compared to the oil-adjuvanted group, it could be questioned whether the adjuvant properties of PLGA/PLA particles in general are too weak to induce similar responses. However, before conclusions are

drawn, further studies on the effect particle stability and surface potential have on antibody responses should be carried out.

Microparticles are superior to nanoparticles in serving as antigen depots

As the depot effect is considered an important adjuvant property of the oil-based formulations used in salmon vaccines, it was of particular interest to investigate the ability of PLGA and PLA particles in retaining antigens after immunization. For this purpose, an isotope labeled protein was encapsulated and injected intraperitoneally. Because nanoparticles were expected to be rapidly absorbed from the peritoneal cavity and possibly cleared from the body, as reported by others [210], sampling was performed at relatively early time points (day 7 and 36) compared to other studies and what could be expected with respect to the depot abilities of the oil-formulation [103;274]. Results from paper II indicated that the overall depot effect of microparticles in fact was superior to nanoparticles, and the latter were found to accumulate antigens in the kidney. Polymer qualities such as MW and LA:GA are known to have an impact on the degradation kinetics of particles [138;139;151;218] and particles were therefore formulated using polymers with high and low MW and various proportions of lactic acid. During long-term *in vitro* incubation at 8^{o}C, nanoparticles released less antigen compared to microparticles irrespective of the mentioned polymer qualities, and based on size, the *in vitro* stability of particles was reversely related to the antigen retention observed *in vivo*. In general, paper II demonstrated that qualities such as high MW of the polymer combined with particle sizes above the phagocytic range are significant factors to increase the ability of particle formulations to depot antigens.

Vaccine efficacy of PLGA nanoparticles

In paper III, the vaccine efficacy of PLGA nanoparticles was evaluated in a challenge test with infectious pancreatic necrosis (IPN), and comparison was made to other vaccine delivery systems, including whole inactivated vaccines, sub-unit vaccines and a plasmid DNA vaccine. Efficacy was evaluated based on parameters such as ability to confer protective immunity and reduce virus dissemination during the infection period following cohabitation challenge. During the immune induction period, nanoparticles provoked high antibody responses compared to the other formulations. Post challenge virus re-isolation from blood and head kidney further demonstrated that infection was established later in nanoparticle vaccinated fish compared to the PBS injected control group. However, at onset of acute

mortality (day 21) infection rates were equal in the control and the nanoparticle vaccinated group, as assessed by immunohistochemistry. Furthermore, despite the fact that the nanoparticle vaccinated fish were able to hold back the virus infection, the survival rates presented as a Kaplan Meyer plot showed that the two nanoparticle vaccines (containing a homologous and a heterologous antigen) clustered with the PBS injected control group, indicating that nanoparticles were unable to confer immunity against IPN. Overall, the nanoparticle vaccinated group performed similar to the pDNA and the subunit vaccines, while the inactivated whole virus vaccines formulated in an oil-based adjuvant generally were superior to all the vaccines in the current study. However, efficacy obtained for the different vaccines are not directly comparable as antigen doses differed between the formulations. For the nanoparticle vaccines, this was due to limiting factors during preparation; high virus titers are needed in the stock solution (W_1-phase) during nanoparticle preparation to yield high antigen loading in particles. Consequently, the antigen dose in the inactivated whole virus vaccine was 200x higher compared to the antigen dose found in the nanoparticle formulations. Unpublished results from studies run parallel to paper III have demonstrated that antigen dose may be a determining factor for protection against IPN after vaccination (with oil-adjuvanted vaccines).

Future perspectives

Factors such as size, surface potential and polymer composition are cardinal determinants for particle-cell interactions following PLGA vaccination and might be important in directing immune responses. Combining these factors with the possibility to co-deliver antigens and non-particulate (receptor-based) adjuvants makes PLGA particles highly flexible in vaccine design. In the current studies, all these aspects have been considered (although in minor detail) and results have demonstrated that PLGA particles are able to induce strong innate responses and serve as antigens depots after immunization of Atlantic salmon. However, their long-term effect on the immune responses seems to be disadvantaged; antibody responses were only transiently up-regulated and the challenge test revealed poor immunity against an intracellular pathogen (all results are summarized in Figure 5).

The fact that microparticles served as excellent antigen depots without this being reflected in antibody responses raises an interesting question; why is the antigen present, but immunologically ignored? Moreover, nanoparticles were able to efficiently transport antigens to an important lymphoid organ and thereby increase the antigen load in a tissue with

abundant macrophage presence. Is this important for the resulting immune response, and if so, how can this quality be exploited to direct responses? As most of the studies presented here were performed *in vivo*, it was made difficult to point out exactly what *particle qualities* are favorable for induction of humoral or cellular responses in salmon. Further studies should address these matters on a more detailed level *in vitro*, aiming to characterize particles that are optimal cellular uptake and for induction of Th1 or Th2 responses.

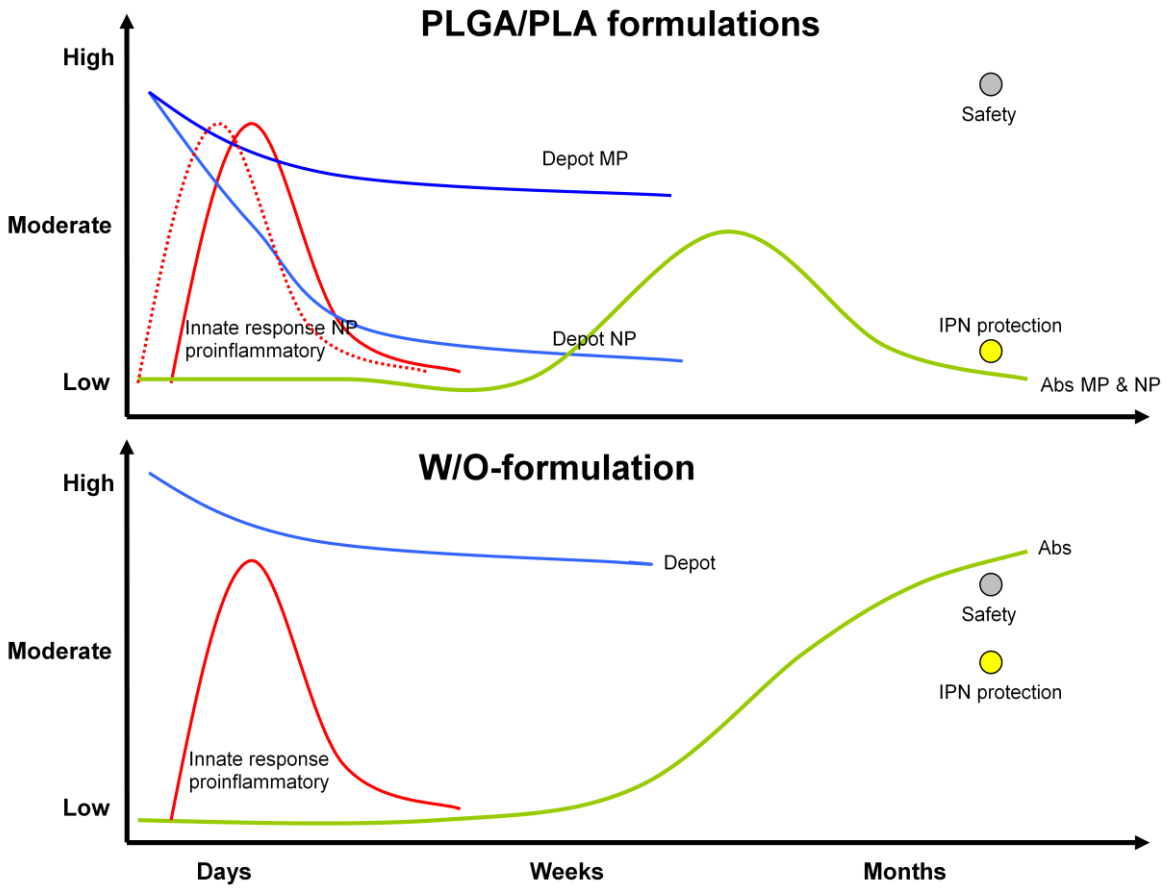

Figure 5. A summary illustration combining all the *in vivo* results obtained in papers I-III. Pro-inflammatory responses (red lines) were found to be equally high in salmon immunized with particle formulations as after immunization with an oil-based formulation, however TNF-α was an exception due to time of induction (red dashed line). The antigen depot (blue lines) was generally better in fish immunized with the oil-based formulation. Microparticles demonstrated moderate depot effect, while nanoparticles had low depot abilities. Salmon immunized with PLGA generally had transient elevated antibody levels (green lines), whereas fish immunized with the oil-based vaccine demonstrated an increasing level of antibodies during the experimental period. The PLGA particle formulations had low scores on the *Speilberg*-scale indicating excellent safety (grey circle), while efficacy (yellow circle) after challenge with IPN was low. The oil-based formulation resulted in a slightly lower safety score, as well as moderate protection against IPN. Abbreviations: NP – nanoparticle, MP – microparticle, Abs – Antibody response.

MAIN CONCLUSIONS

- PLGA nanoparticles carrying antigens and immunomodulators are able to induce strong innate responses in Atlantic salmon.
- PLGA particles induce elevated, but transient antibody responses after immunization.
- PLGA nanoparticles were unable to induce protective immunity against IPN.
- PLGA microparticles may serve as antigen depots at the injection site.
- PLGA nanoparticles increase the antigen load in the head kidney.
- PLGA particles made of polymers with high molecular weight are superior to low molecular weight polymers for *in vivo* antigen retention.

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* The undersigned is familiar with the fact that *Vibrio salmonicida* is now called *Aliivibio salmonicida*, but the former name was used as the preface is a historical look back.

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PAPER I

PAPER II

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PAPER IV

PAPER V

ISBN xxx-xx-xxxx-xxx-x