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Nanoparticles and microparticles of polymers and polysaccharides to administer fish vaccines

Andrea Rivas-Aravena, Ana María Sandino and Eugenio Spencer

Laboratorio de Virología, Centro de Biotecnología Acuícola, Universidad de Santiago de Chile.

ABSTRACT

Aquaculture has become an important economic sector worldwide, but is faced with an ongoing threat from infectious diseases. Vaccination plays a critical role in protecting commercially raised fish from bacterial, viral and parasitic diseases. However, the production of effective vaccines is limited by the scarcity of knowledge about the immune system of fish. Improving vaccines implies using antigens, adjuvants and employing methods of administration that are more effective and less harmful to the fish. In this context, in recent year there have studies of methods of encapsulating antigens in matrices of different types to apply in fish vaccines. This work reviews the new methods to improve fish vaccines by encapsulating them in polymers and polysaccharides.

Key words: Nanoparticles, vaccine, aquaculture.

INTRODUCTION

The use of vaccinations is essential in aquacultural production in order to keep fish free of bacterial, viral and parasitic pathogens. This results in an ongoing need for research into new vaccines and especially new means of applying vaccines that are fish-friendly while at the same time strengthening the effect of the vaccination. However, until now vaccines have not been developed that provide efficient protection, especially to prevent viral, intracellular bacterial and parasitic infections (Hastein et al., 2005; Sommerset et al., 2005). Added to this, the great majority of vaccines are currently administered in water/oil formulas that allow for an extended release of antigens (AG) while protecting them from degradation and inducing an inflammatory effect that strengthens the response of antibodies against the vaccine. However, despite the benefits that they can provide, adjuvants can cause adverse effects such as accumulation of myelin, adherence to tissue (Midtlyng 1997; Poppe & Breck. 1997), deformation of the skeleton (Berg et al., 2006), granulomatous peritonitis, growth inhibition and increased mortality rates (Koppang et al., 2005; Evensen et al., 2005), as well as problems of autoimmunity (Koppang et al., 2008). Given the problems associated with the vaccines used in fish farming, it is critical to investigate and develop new vaccines, adjuvants and methods of release that are more efficient in protecting fish without producing negative effects.

In general, assessing vaccines involves measuring their effectiveness in stimulating immune response by evaluating antibodies in the blood of vaccinated fish. However, this humoral response often does not correspond to the level of protection provided by the vaccine (Cuesta et al., 2010; De Las Heras et al., 2010; Dorson et al., 1978; Gomez-Casado et al., 2011; Koellner y Kotterba 2002). The increased expression or the synthesis of some molecules associated with stimulating innate immune response in fish such as some inflammatory cytokines, interferon (IFN) and associated molecules is also currently used as a measure of the efficacy of vaccines (Walden et al., 1986; Walsh et al., 2003; Wang et al., 2011; Wang et al., 2005).

The above is not consistent with what has been found in recent studies about the design of vaccines for mammals sholwed the importance of allowing the antigen to interact efficiently with antigen-presenting cells (APC) to generate a favorable cellular and humoral immune response against the pathogen. Recognition and presentation by APC activates the specific and innate immune response of the individual. In higher mammals APCs include dendritic cells (DC), macrophages and B cells; their activation leads to presenting the antigen to immune system cells, activating the cellular response of lymphocyte helpers (Th), cytotoxic lymphocytes (CTL) and lymphocytes B. Phagocytized extracellular antigens are presented by membrane molecules called major histocompatibility complex class II (MHC class II) to Th lymphocytes, which leads to activation of lymphocytes B for the synthesis of specific antibodies against the antigen. The intracellular antigens that are not phagocytized are presented by MHC class I, which is recognized by CTL and is capable of lysing the infected cells. In this way, antigen recognition presented by APC through MHC I and II and the maturing of dendritic cells leads to the activation and orchestration of all levels of the immune response of the individual (Banchereau & Steinman 1998; Banchereau et al., 2012). It has also recently been demonstrated that stimulating APC with inactivated phagocytized pathogens generates a cross-presentation of antigens by MHC I and II, triggering the immune response mediated by APC in all its levels (Brode & Macary 2004; Joffre et al., 2012; Rodriguez-Pinto 2005; Wetzel & Parker 2006). This is important for the use of vaccines based on inactivated pathogens and others that are not endogenous antigens of the APCs (Pooley et al., 2001; Sigal et al., 1999).

There are several reports of characterization of macrophages and DC presenters of antigens in fish with phagocytic activity and production of proinflammatory cytokines (Bassity y Clark 2012; Haugland et al., 2012; Lugo-

Corresponding author: Andrea Rivas-Aravena. Laboratorio de Virología, Centro de Biotecnología Acuícola, Universidad de Santiago de Chile. Av. Bernardo O'Higgins 3363, Estación Central, Santiago, Chile. Phone: 27181115 Email: Andrea.rivas@usach.cl

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Villarino et al., 2010; Pettersen et al., 2008; Vallejo et al., 1992). It has also been determined that B cells in fish have phagocytic activity (Li et al., 2006), which, as has been shown recently in mammals, can strengthen APC activity (Parra et al., 2012).

Vaccines are administered to fish by intraperitoneal or intramuscular injection. While injection allows for the use of smaller and precisely known doses of AG and confers protection for longer periods of time, the technique requires more manipulation of the fish and the use of anesthesia, which causes stress and greater harm to the fish, along with high costs for the producer. Consequently, under field conditions fish weighing over 20 g are vaccinated while smaller fish, which are more vulnerable because they have not yet fully developed their immune system, are left unprotected. Laboratorios Centrovet Ltd. in Chile is the only company that has developed oral vaccines encapsulated in MicroMatrix® (licensed under Advance BioNutrition Corp), which have had good results against salmonid rickettsial septicemia (SRS) and Infectious salmon anemia virus (ISAV). Several studies have shown that the administration of antigens or immunostimulants orally or by immersion can stimulate the immune response associated with the intestine of the fish (Quentel & Vigneulle 1997; Rombout et al., 2006). In fish, as in higher vertebrates, there is very complex mucosa-associated lymphoid tissue (MALT) in the gut (GALT), skin (SALT) and gills (GIALT), with a specific and specialized immune response for these tissues, with different immune cells and antibodies from those found in the blood (Tyagi et al., 2012). The efficiency of this response depends on the immunization route, given that oral and anal administration in fish activates GALT, which presents greater efficiency in some species. Immersion activates SALT and GIALT, while injection generally does not activate any of these (Salinas et al., 2011; Joosten et al., 1997; Rombout et al., 1989). Parallel to this, it has been shown that the enterocytes in the hindgut are capable of transporting antigens and that in the lamellae and epithelial cells there are populations of macrophages and lymphoid cells that can participate in presenting antigens in the intestine of the fish (Doggett & Harris 1991; Georgopoulou & Vernier 1986; Rombout et al., 2006). All of this indicates that the administration of the vaccine orally or by immersion stimulates a different response from that stimulated by intraperitoneal or intramuscular administration, one that is similar to the response to the pathogen.

All of the above indicates that to obtain a good vaccine it is critical to use antigens and adjuvants that stimulate the appropriate immune response and that permit forms of delivery of the antigen that are not harmful to the fish. Consequently, in aquaculture, as in other forms of animal production, alternative systems of administering antigens are being explored that strengthen the vaccine, such as encapsulation of molecules in polymeric and polysaccharide particles.

Microparticles (MP, size in μm) and nanoparticles (NP, size in nm) have emerged as efficient and safe methods to deliver drugs and antigens to animals (Toyokawa et al., 2008). In fact, at present particles that encapsulate different types of molecules are administered to humans (Food and Drug Administration, FAO; Lu et al., 2009). Polymeric and polysaccharide particles can be designed to be administered by different means, such as orally or by injection or inhaling, and to encapsulate different types of molecules (Morachis et al., 2012). They can also be directed to specific sites of interest in the organism while protecting them from biological degradation (Morachis et al., 2012). The direct benefit of encapsulation in particles is that it permits reducing the doses of medication, making its delivery to specific tissue and avoiding or reducing the toxic effects or secondary effects of the drug, making the delivery of the drug more efficient and less expensive (Toyokawa et al., 2008). The small size of nanoparticles makes it possible to spread them throughout the organism through the circulatory system, including the capillaries, finally entering the target cells. Larger microparticles can release antigens in the place of administration (circulatory system, gastrointestinal tract), allowing the synthesis of antibodies against the antigen and a general inflammatory response (Tafaghodi et al., 2007).

The particles used to deliver molecules to living organisms are biodegradable and non-toxic and can be designed to be highly versatile in terms of their size, physical-chemical properties and the release of antigens; it is possible to modify them for the efficient delivery of any type of molecule in specific organs, tissues or cells. For example, nanoparticles can be designed to change the physical-chemical properties of the drug that is being administered, increasing its solubility, decreasing its elimination time, improving its renal excretion or varying the selection and mechanism of incorporation in cells or its distribution in the organism (Doshi et al., 2010; Morachis et al., 2012). The polymers are also versatile in their facility to join with antibodies and other molecules that interact with cellular receptors, such as DNA aptamers (Morachis et al., 2012).

Many polymeric and polysaccharide particles have adjuvant capabilities because they protect the encapsulated molecule from biological degradation, stimulating the immune response against the antigen (Broos et al., 2010; Clawson et al., 2010). This form of delivery also allows the antigen to reach the necessary organs and tissues to stimulate an efficient immune response, which is difficult with soluble antigens regardless of the method of administration. A nanoparticle can be the same size as a pathogen and can be easily incorporated by the APC, stimulating the immune response (Audran et al., 2003; Diwan et al., 2003; Elamanchili et al., 2007; Fischer et al., 2009). It has been reported that nanoparticles are mainly endocyted while microparticles are mainly phagocyted (Burgdorf & Kurts 2008). Other reports indicate that APCs easily phagocyte nano and microparticles (between 150 nm to 4.5 μm, (He et al., 2010; Thiele et al., 2001), the optimal size for phagocytosis being 500 nm (Burgdorf & Kurts 2008). It has also been shown that microparticles have more affinity for peripheral dendritic cells, while nanoparticles prefer dendritic cells lodged in lymphatic nodes (Manolova et al., 2008; Reddy et al., 2006). It has also been shown that microparticles of less than 10 μm are incorporated into the intestine, nanoparticles of more than 100 nm are incorporated in monocytes and those of less than 100 nm can be incorporated in non-phagocytic cells (Agnihotri et al., 2004; Desai et al., 1997). In this manner, nanoparticles that encapsulate antigens can resemble pathogens in terms of the means of administration, mirroring the route of pathogen entry and the immune response that is triggered (Gutierro et al., 2002). The particles may also have antigens on their surface, which is a good stimulant for the B cells (Fehr et al., 1998; He et al., 2005).

Methods are now being developed in aquaculture to encapsulate antigens for their efficient and harmless

application while strengthening the effect of nano- or microparticle vaccines. The first tests of encapsulating antigens against *Vibrio anguillarum* in fish through the use of prills, acid resistant coating, dextrose and resistant films did not stimulate antibody production or result in better protection than that offered by inactivated naked bacteria (Lillehaug 1989; Wong 1992). Nevertheless, new technologies of encapsulation of antigens for fish have been implemented, such as those employing polymers and polysaccharides.

METHODS OF ANTIGEN ENCAPSULATION

Alginate

Alginate is a copolymer of β-D- mannuronic acid (M) and α-L-guluronic acid (G) that is found in the cell wall of brown algae. Alginate has been used widely in encapsulation of antigens for several reasons: its low level of toxicity (Lim & Sun 1980); its mucoadhesiveness allows contact of the alginate particle with the walls of the epithelial mucus (Wee y Gombotz 1998); it is resistant to acidic conditions and to proteases; and it is inexpensive (Bowersock et al., 1999). The characteristics of the alginate particle are a result of its size, antigenic composition, the strategy in its production, the choice of alginate and the antigen concentration. For example, the production of microparticles from alginate is generally done by emulsification/gelation, where the alginate emulsion in the presence of the molecule to be encapsulated reacts with calcium ions that promote the piling of G. In this technique, the specifications of the protocol, that is, the viscosity, molecular weight and the concentration of the alginate, stirring times, temperature, gelation agent, the arrangement of its monomers (MM, GG and MG form), type of surfactant, among others, markedly influence the physical-chemical characteristics obtained (Lemoine et al., 1998; Rodrigues et al., 2006). Similarly, molecules are mainly released by diffusion or erosion of the microparticle, which depends on the molecular size of the active agent and the concentration of the alginate, as well as the external environment (Tanaka et al., 1984).

Notably, there have been several reports indicating that alginate can act as an antigen adjuvant (Borges et al., 2008; Tafaghodi et al., 2007). Administered alone or as a complement, alginate is capable of increasing the survival and weight of fish (Fujiki et al., 1994; Yeh et al., 2008). The administration of alginate has been shown to stimulate the immune response of carp (*Cyprinus carpio L.*) and the brown-marbled grouper (*Epinephelus fuscoguttatus*, Cheng et al., 2008; Huttenhuis et al., 2006; Yeh et al., 2008) and increases the protection of the turbot (*Scophthalmus maximus L.*) against *V. anguillarum* (Skjermo & Berghb 2004; Skjermo et al., 1995), and the orange-spotted grouper (*Epinephelus coioides*) and brown-marbled grouper against iridovirus and *Streptococcus sp*. (Cheng et al., 2008; Yeh et al., 2008).

One of the characteristics of alginate microparticles that makes them useful for delivering drugs is their resistance to acid pH, which impedes the release of the antigen in the stomach of the fish (pH 2-4) and favors their its release in the foregut or hindgut (pH 7 and 8.3, respectively) (Joosten et al., 1997; Leal et al., 2010; Rodrigues et al., 2006; Salinas et al., 2011). Microparticles from 10 to 50 μm can be efficient for vaccinating, although it has been shown that the smaller the size, the greater the efficiency of incorporation (Rodrigues et al., 2006; Romalde et al., 2004). There are reports of administering antigens and molecules to fish enveloped in alginate with microparticles of 1 to 50 mm, as will be described in detail below.

Bacterial antigens encapsulated in alginate

There have been several studies of the use in aquaculture of bacterin encapsulated in alginate. The results vary with the fish species, method of delivery and use of a booster, but in general they are not related with stimulation of fish antibodies. In fact, the oral administration of microparticles of *Flavobacterium* to Nile tilapia (*Oreochromis niloticus*) did not stimulate the production of antibodies in serum, but intraperitoneal and intramuscular administration did. This stimulation does not correlate with protection against infection with any of the studied vaccines (Leal et al., 2010). However, better results were obtained when bacterin microparticles were used as an oral booster after intraperitoneal vaccination with naked bacterin, reaching 87% relative survival (RPS) (Altun et al., 2010; Romalde et al., 2004). Oral administration to goldfish (*Carassius auratus*) of alginate microparticles of the protein A of *Aeromona salmonicida*, alone or fused with membrane translocation sequences (MTS) derived from Kaposi fibroblast growth factor, stimulated antibodies in serum a month after administration for three days to a week, and after two months when it was administered for five days to a month, which correlated with the presence of protein A in the serum of vaccinated fish. There was greater stimulation of antibodies in serum with the intraperitoneal administration of a booster two weeks after the oral administration of the vaccine. Despite the increase in antibodies, all the vaccinations caused only a slight decrease in the symptoms among the infected fish (Maurice et al., 2004)

The oral and intraperitoneal administration of *Lactococcus garvieae* microparticles to rainbow trout (*Oncorhynchus mykiss*) protected against infection, reaching a relative survival of 50%, which was less than the survival rate obtained with naked bacterin applied intraperitoneally (83.3% RPS) (Altun et al., 2010; Romalde et al., 2004). This is consistent with the fact that in general vaccines against gram positive pathogens *lactococci* and *Streptococcus iniae* are only effective when administered intraperitoneally, although the efficacy is for short periods (2-3 months, Eldar et al., 1997; Romalde et al., 1999).

The different means of administering vaccines stimulate different types of immune response in carp (*Cyprinus carpio)*, similar to what occurs in mammals. Microparticles of bacteria such as *Vibrio Anguillarum* administered orally increase the number of antibodies in the mucus of the gut and gills and very little in serum. In contrast, antibodies increased in serum, head kidney, blood, but not in the gut and gills in carp vaccinated and challenged intramuscularly (Joosten et al., 1997). Although in the case of carp the vaccination with microparticles is capable alone of increasing the number of antibodies in serum, with rainbow trout a booster is required to do the same. This is probably due to the difference in swelling and antigen release from alginate microparticles at different pH levels, resulting in the microparticles not releasing antigens in the stomach but rather in the intestine. Unlike the rainbow trout, the carp does not have a stomach, so that antigens are released sooner and likewise stimulate an immune response more rapidly. In fact, antigens are found carp enterocytes 16 hours after administering the

vaccine, while they are found in the enterocytes of rainbow trout 24 hours after administration. Finally, the response of immunoreactive macrophages is only found at 36 hours post-administration (Joosten et al., 1997).

Viral antigens encapsulated in alginate

Vaccinations with alginate microparticles against virus have used DNA vaccines that code for immunogenic viral protein. Encapsulation has resulted in obtaining microparticles smaller than those of bacteria (10 um), which allows for their incorporation and DNA expression in organs such as spleen, kidney, liver, pyloric caeca and stomach for months after the vaccination by depositing microparticles in the stomach using stents (De Las Heras et al., 2010; Tian et al., 2008). The period of protection is much longer than administration of naked DNA is administered (Tian et al., 2008; Zheng et al., 2006).

The oral vaccination of Japanese flounder (*Paralichthys olivaceus*) with microparticles of DNA that encodes for a fragment of the protein of the capsid the lynphocystis disease virus (LCDV) (Zheng et al., 2006) protected against viral disease and produced a sustained increase in specific antibodies against LCDV in serum for up to 14 weeks, at which point it began to decline owing to the degradation of the DNA. In contrast, fish vaccinated with naked DNA did not produce antibodies, which suggests that the alginate particles that enter the blood are distributed to immune tissue and stimulate immune response for longer periods than does naked DNA (Tian et al., 2008).

The oral vaccination of rainbow and brown trout (*Salmo trutta*) with microparticles of DNA of the VP2 protein of the infectious pancreatic necrosis virus (IPNV) stimulated the IFN and Mx at 7 and 5 days post-vaccination (dpv), respectively, and increased neutralizing antibodies against IPNV during the eight weeks of evaluation, peaking at three weeks. In this case, the protection against IPNV infection was high, with a 15% mortality rate for brown trout and 15-20% for rainbow trout. Moreover, the unvaccinated fish that survived the infection present the virus circulating, which can be isolated with high titers in a first passage in BF-2 cells, while the IPNV titers obtained from the surviving fish that were vaccinated with microparticles were 1 to 7 orders of magnitude lower and were obtained in the second passage with BF-2 cells, showing that there is less risk that vaccinated carriers will spread the disease than unvaccinated carriers (De Las Heras et al., 2010).

These studies have shown that the oral administration of bacterins encapsulated in alginate provides the same or less protection than that provided by intraperitoneal administration of naked bacterins and that the vaccination of viral DNA encapsulated in alginate provides protection against viral diseases. Consequently, encapsulation with alginate is promising in terms of providing protection against viral and bacterial pathogens in aquaculture.

Chitosan

Chitosan is a linear polysaccharide compound of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine that is derived from the deacetylation of chitin, which is found in the exoskeletons of crustaceans, insects and some microorganisms. Deacetylation changes the chain conformation and electrostatic properties of chitin, increasing its solubility and leaving amine-reactive groups in its ends, from which derivatives can be obtained with specific characteristics by adding different molecules at the ends. Antimicrobial, anti-inflammatory, hemostatic and antiviral properties have been attributed to chitosan. It has also been attributed to inhibit tumors, regenerate tissue, heal injuries and to be an immunostimulant. Chitosan is mucoadhesive and increases transcellular transport in intestinal cells (Davis 2006; Ghendon et al., 2008; Ouji et al., 2002).

Chitosan has adjuvant characteristics similar to those of Freund's incomplete adjuvant and better than those of aluminum hydroxide in terms of its capacity to increase the protection offered by vaccines for mammals (Coeshott et al., 2004; Ghendon et al., 2008; Zaharoff et al., 2007). In fact, chitosan stimulates the synthesis of cytokines and activates immune cells such as macrophages, natural killer cells (NK), APC cells and T lymphocytes (Foged et al., 2005; Nagamoto et al., 2004; Nishimura et al., 1984; Peluso et al., 1994; Porporatto et al., 2005; Koppolu & Zaharoff 2013).

Chitosan particles have highly variable characteristics depending on their purity, molecular weight, degree of deacetylation, quality and viscosity. Also, a variety of particle sizes can be obtained (Koppolu & Zaharoff 2013). In general, low molecular weights of chitosan ($1.5^* 10^5$) produce stronger capsules. The release of encapsulated content depends on the diffusion among the pores and the chitosan charge, given that acid molecules are more permeable. The size of the particle is related to the type of incorporation into the organism (Agnihotri et al., 2004).

The beneficial effects on fish of chitosan have been demonstrated. Diets supplemented with 0.25 g of chitosan/Kg for rainbow trout improve resistance to environmental stress and hematological parameters, resulting in higher levels of lymphocytes and decreased levels of neutrophils and eosinophils (Meshkini et al., 2012). It has also been demonstrated that the DNA of β-galactosidase encapsulated in chitosan leads to significantly greater expression of the enzyme in the spleen, stomach and gills in *Tilapia* when the vaccine is administered orally than by injection (Ramos et al., 2005).

DNA fish vaccines encapsulated in chitosan are viable and stable because DNA is easily incorporated into chitosan, given its positive charge. The microparticles protect the encapsulated DNA from degradation by DNase (Rajesh Kumar et al., 2008) and from shear forces (Tian et al., 2008). Notably, the DNA vaccine can transfect fish cells and present very low levels of cytotoxicity (Rajesh Kumar et al., 2008). Thanks to these characteristics, chitosans can be used to administer DNA and vaccines of diverse natures to fish orally or by injection. These characteristics have led to studying the application of chitosans in the context of developing vaccines for fish.

Bacterin antigens encapsulated in chitosan

The DNA that encodes for the protein of the external membrane of 38kDa (OMP38) of *Vibrio anguillarum* was encapsulated in chitosan and administered with feed to Asian sea bass (*Lates calcarifer*). The viral protein was detected in gills, spleen, liver and intestines. Three weeks after the vaccination, there was a low level of stimulation of antibodies of the fish. The protection against *Vibrio anguillarum* caused a 46% RPS (Rajesh Kumar et al., 2008). Also, when chitosan/alginate microparticles were used to encapsulate *Vibrio anguillarum* or BSA (bovine serum albumine) it was found

that more antigen is released with lower molecular weight of chitosan and that as with alginate, there is more antigen release in a basic (pH 9) rather than an acid environment (pH 3, Polk et al., 1994).

Viral antigens encapsulated in chitosan

Encapsulating the DNA of a fragment of the protein of the LCDV capsid (Zheng et al., 2006) produces microparticles of the same size (10 μm) as those of alginate using a similar strategy to water/oil emulsion (Tian et al., 2008) and with stability levels comparable to those of microparticles of alginate in the acids of the stomach and the intestine (pH=2 and 9, respectively). Following the administration to rainbow trout by stomach intubation, the expression of the protein fused to GFP was detected in gills, intestine, spleen and kidney for 90 days, and was less intense than when alginate microparticles were used (Tian et al., 2008). Antibodies were stimulated to the same magnitude as with alginate microparticles (Tian et al., 2008), but with a maximum in week four and a slow decline to week 16 without reaching the level of the negative control, indicating that there is a prolonged synthesis of antibodies against LCDV in vaccinated fish (Tian et al., 2008).

Despite the apparent advantages offered by chitosan, there have not been sufficient studies on the effectiveness of encapsulating antigens with chitosan.

Liposomes

Liposomes are vesicles of phospholipids that form spontaneously in aqueous solutions and are capable of trapping dissolved particles in such solutions. They are biodegradable, liberating the charged molecule slowly when they degrade in the organism. Liopsomes can have different characteristics depending on their size, the number of lamellae that make them up and the capacity to trap molecules in solution (Gregodiaris 1990).

Liposomes have good immunogenic capacity and have been approved for use as adjuvants with humans. It is argued that their rapid incorporation in APCs in the injection site permits them to act as a good adjuvant, activating the cellular response. Their versatility allows for encapsulating molecules of different characteristics and of different types (Gregodiaris 1990; Gregoriadis et al., 2002; Mbow et al., 2010).

Beneficial effects have been determined from administering liposomes derived from lecithin egg yolks as a food supplement for larvae of gilthead seabream (*Sparus aurata*) and white grouper (*Epinephelus aeneus*) (Koven et al., 1999). Liposomes have been used in different types of research related to fish farming. Clodronate liposomes have been used to investigate macrophage activity in rainbow trout (Espenes et al., 1997) and phosphatidylcholine liposomes have been used successfully to transfect African carp embryos by incubating the embryo with the liposome at room temperature, achieving high levels of efficiency in transfecting the DNA that encodes for neomycin (92%, Szelei et al., 1994).

The liposome charge needs to be considered when administering molecules to fish in liposome, given that fish gills contain a high level of mucin. The sialic acid of the mucin is deprotonated by the pH of the water (Lumsden & Ferguson 1994), facilitating its interaction with cationic liposomes of <100nm that encapsulate DNA, for example, increasing the residence time and the uptake of the load. This interaction results in high concentrations of cationic liposomes that are fatal for fish, presumably because the interaction with the gills can provoke hypoxia in the fish. In contrast, similar concentrations of anionic or neutral liposomes are not fatal (Romoren et al., 2002).

Fernández-Alonso et al. (1999) showed that liposomes could allow for DNA expression when they are administered to fish. The immersion of rainbow trout in 10-20 μm of DOTAP liposomes containing codifying DNA for green fluorescent protein (GFP) resulted in detecting GFP in the fins of 0.2-0.5-gram fish, suggesting that vaccination by immersion is feasible for small fish (Fernandez-Alonso et al., 1999). They also demonstrated that the encapsulation in DOTAP of the DNA that encodes for GFP expresses the protein in the caudal fin of rainbow trout at a similar level to that when the fish are exposed to short ultrasound pulses in the immersion bath of naked DNA (Fernandez-Alonso et al., 2001). Studies have indicated that there is no direct relationship between the size of the liposomes and the specific organs in which they accumulate, but there is a relationship in terms of their capacity to lodge in certain organs. When large unilamellar phosphatidylcholine (PC) liposomes (LUV, 250 nm) were administered interperitoneally to rainbow trout they accumulated in greater proportions in organs than did multillamellar liposomes (MLV, 1-5 μm). At 24 hours after administration, they had accumulated (in decreasing order) in the spleen, head kidney, posterior kidney, visceral fat and liver, and were almost indistinguishable in gills, heart and muscles (Power et al., 1990). The accumulation of liposomes in hematopoietic organs has also been documented by (Nakhla et al., 1994). Liposomes are easily degraded in the stomach, so that studies of vaccinations of fish with liposomes involve administration by IP injection or immersion.

Bacterin antigens encapsulated in liposomes

Phosphatidylcholine liposomes, which encapsulate a mixture of inactivated *Aeromona salmonicida* with formaline, together with LPS and inactivated toxin were administered by immersion to rainbow trout and provided limited protection against furunculosis, being slightly more efficient than the free antigen (Rodgers 1990). Intraperitoneal vaccination with LPS in MLV and LUV liposomes followed by the booster with LPS had more effect in stimulating antibodies and for a longer period of time than the vaccination and booster with LPS. Antibody stimulation was greater depending on the number of immunizations, without showing significant differences when using different charge of phospholipids (Nakhla et al., 1997)

Viral antigens encapsulated in liposomes

The administration by immersion of particles of DOTAP of the DNA that encodes for the G protein of VHSV allows for a similar expression in the caudal fin of rainbow trout as when the particles are administered by short ultrasound pulses in the immersion bath of naked DNA, resulting in an increase in the number of antibodies of a lower magnitude than when administered by ultrasound or IP. However, this expression of DNA does not offer protection from VHSV infection,

while ultrasound and IP administration do offer protection (Fernandez-Alonso et al., 2001)

More research is needed into liposomes of different types to determine their utility in the encapsulation of antigens for fish. Modified liposomes that are resistant to digestion in the stomach make it possible to use oral vaccines.

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

PLGA is a poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) that can be designed in different sizes and shapes and can be used to encapsulate many types of molecules. PLGA is easily degraded in the organism, since in an aqueous environment its ester bond is hydrolyzed, which allows for the easy release of antigens. The velocity of hydrolysis in water is variable and depends on the PLA/PGA ratio of the polymer, but it is higher in acid environments. Unlike PGA, PLA contains a methyl group, and at higher proportion of PLA the copolymer is more hydrophobic, slower the hidrozylation. Similarly, the PGA/PLA ratio influences other physical-chemical parameters of the particles such as mechanical force, hydration capacity (swelling behavior), gelation temperature, charge, etc. PLGA has a negative charge.

PLGA particles show great flexibility in the encapsulation of different molecules (Elamanchili et al., 2007; Tamber et al., 2005) and have been used to administer vaccines to humans and other mammals (Lu et al., 2009). PLGA particles can act as adjuvants (Diwan et al., 2004; Katare & Panda 2006) and their use for humans and in veterinary medicine has been accepted by the FDA. It has been demonstrated that PLGA microparticles generally promote a humoral response, while PLGA nanoparticles trigger a cellular response (Chong et al., 2005; Gutierro et al., 2002; Harding & Song 1994).

PGLA particles allow for the co-release of multiple antigens and are capable of transporting antigens to intracellular compartments. As they are less hydrophilic than alginate particles, they are easily incorporated into the cell. They can be designed to be same size as the pathogens to stimulate APC (Broos et al., 2010; Diwan et al., 2004; Gupta et al., 1997; Hamdy et al., 2007; Johansen et al., 1998; Katare et al., 2005; Norton et al., 2010; Schlosser et al., 2008). Also, depending on their characteristics, their biological degradation can take months to years (Prokop & Davidson 2008; Vert et al., 1994).

In general, PLGA encapsulating particles are obtained by water/oil/water emulsion in which the PLGA is dissolved in an organic solvent such as chloroform, dichloromethane or ethyl acetate and is emulsified with an aqueous solution of AG in the presence of a surfacant that is emulsified again in an aqueous solution, generally of polyvinyl alcohol. The method of obtaining the copolymers, their size and composition are critical characteristics required of PLGA particles. During the protocol, sonication results in nanoparticles of ±100 μm, homogenization results in particles of ±1 μm, and vortexing/stirring in particles of ±10 μm. Also, copolymers rich in PLA are more stable to hydrolysis (Desai et al., 1996).

PLGA nanoparticles can be modified according to the characteristics that it is desired to strengthen. For example, PLGA nanoparticles can generally resist the environment of the gastrointestinal tract when administered orally (Li et al., 2008; Pandey & Khuller 2007; Shaikh et al., 2009). Nevertheless, to increase the stability in acid environments a strategy has been developed of polyethylene glycol coating (PEG, Garinot et al., 2007) that decreases interaction with the environment and offers protection from the medium and macrophages (Owens & Peppas 2006). The particles are also coated with the anionic polymer Eudragit L30D, which impedes hydrolysis in acid pH but does permit hydrolysis in basic pH (Naha et al., 2008). Once inside the cell the PLGA nanoparticles can escape from endosomes (Panyam et al., 2002), facilitating the presentation of antigens encapsulated by cross-presentation pathway (Shen et al., 2006). PLGA nano and macroparticles may also be conjugated with proteins, antibodies or aptamers that allow selecting particles by specific cell types (Singh / Srivastava 2003).

In mammals PLGA nanoparticles accumulate rapidly in the liver, bone marrow, lymphatic nodes, spleen and peritoneal macrophages (Makadia & Siegel 2011). Notably, when PLGA nanoparticles are administered orally in mammals they can increase the immune response associated with mucosal membrane (Danhier et al., 2012). They can also be phagocytized by APC much more easily than soluble antigens, thus imitating the nature of the pathogens. In fact, PLGA nanoparticles have been used to incorporate antigens into human dendritic cells, demonstrating that PLGA nanoparticles cause a significant increase in CTL activity (Ma et al., 2012), Th activity, the expression of regulatory cytokines and the maturation of dendritic cells. Notably, PLGA nanoparticles can activate dendritic cells in the absence of antigen, stimulating the expression of cytokines, immune cells and co-stimulatory molecules (Clawson et al., 2010; Cruz et al., 2011; Diwan et al., 2003; Elamanchili et al., 2004; Goforth et al., 2009; Gutierro et al., 2002; Hamdy et al., 2011; Lutsiak et al., 2002; Manolova et al., 2008). These characteristics of PLGA significantly increase the immunogenic capacities of encapsulated antigens.

After incubating different-sized PLGA particles with the TO leukocyte line of Atlantic salmon (*Salmo salar*), nanoparticles are incorporated more than microparticles in cellular cytoplasm (Fredriksen & Grip 2012). Also, using media that simulate the environment of the fish gastrointestine, it has been shown that the release of DNA is more rapid with large-sized PLGA nanoparticles. For nanoparticles of <100nm, less than 10% of DNA is released in a medium that simulates the gastric fluid and less than 6.5% in medium that simulates intestinal fluid in the first 12 hours (Tian et al., 2008), while the release of DNA from nanoparticles of <330nm is rapid, 100% in 60 h at pH 2, and in 90 h at pH 9 (Tian & Yu 2011).

PLGA particles can be administered orally to fish. Being hydrophobic, PLGA particles swell when exposed to solutions, which occurs mostly in acids, allowing for the release of the molecular load. Experimenting with different PLG: PLA ratios, found that the ratio 75:25 stabilized the nanoparticles and slowed their degradation in the gastrointestinal tract (Fredriksen & Grip 2012).

PLGA microparticles are capable of protecting antigens, increasing the retention time in the stomach and slowing down the passage to the intestine of the Atlantic salmon (Lavelle et al., 1997), allowing antigens to remain for extended periods in sera and organs (O'donnell et al., 1996). Other studies have shown that 550-nm PLGA nanoparticles encapsulating coumarin-6 accumulate more in the hindgut than the foregut (Adomako et al., 2012).

It has been shown that nano- and microparticles of PLGA encapsulating AG administered by intraperitoneal injection have the adjuvant capacity to stimulate the expression of pro-

inflammatory cytokines and antibodies in a similar manner to that of oily adjuvants in rohu (*Labeo rohita*) and Atlantic salmon (Behera et al., 2010; Fredriksen & Grip 2012; Fredriksen et al., 2011). The immune response is stimulated in Atlantic salmon within two days after intraperitoneal administration of PLGA nanoparticles of β-glucan. Administering β-glucan and an adjuvant in nanoparticles generates an inflammatory response in the spleen and kidneys, similar to or greater than that provoked by oily adjuvants at two days post-treatment. It is notable that the response, although in a lesser magnitude, is obtained when the nanoparticles are used alone. The response is more pronounced in the spleen, where the nanoparticles are reported to accumulate (Fredriksen et al., 2011). Respiratory burst activity (RB), myeloperoxidase activity and other immune parameters were also higher with the use of nanoparticles than with the use of oily adjuvants (Behera et al., 2010), and the response of antibodies was similar to that of oily adjuvants (Behera et al., 2010; Fredriksen & Grip 2012). By 60 and 75 days after administration the nano- and microparticles of gamma globulin from human blood and β-glucan stimulated high levels of antibodies, up to 90 and 120 days, respectively. This response is less prolonged when the antigen is administered in an oily adjuvant (Fredriksen y Grip 2012). The size of the PLGA particles markedly affects where they will accumulate. PGLA microparticles (8.1μm) and the formulation of oily adjuvant form deposits in the injection site and adjacent organs, while PLGA nanoparticles (<1000nm) accumulate primarily in the kidneys. This suggests that microparticles release antigens locally in the injection site, while nanoparticles associate with macrophages that can phagocyte them to present antigens (Fredriksen y Grip 2012). The intrabuccal administration of human gamma globulin (HGG) PLGA stimulates antibodies in serum but not in cutaneous or intestinal mucous or in bile (Lavelle et al., 1997).

PLGA has been among the most versatile polymers for encapsulation in terms of allowing for modifications and improvements that have been tested in mammals. In relation to vaccinating fish, PLGA has been the most extensively studied method of encapsulating antigens, with the successful application of nano- and microparticle vaccines that have offered effective protection against pathogens, as will be described below.

Bacterial antigens encapsulated in PLGA

In general, bacterin encapsulation in PLGA has yielded good results when administered intraperitoneally. The bacterin of *Lactococus garvieae* encapsulated in PLGA and administered orally provided the same magnitude of protection to rainbow trout as that encapsulated in alginate (62.79% RPS) and to a lesser degree than bacterin administered by IP (95.34% RPS) when the challenge was presented at 30 days post-vaccination. However, when the vaccinated fish received a booster with PLGA or alginate with bacterin, the survival rate was double that of fish that did not receive the booster (Altun et al., 2010).

Recombinant outer membrane protein (OMP) of *Aeromonas hydrophyla* encapsulated in 1.121-μm microparticles of PLGA can induce an innate immune and antibody response in rohu when administered intraperitoneally (Behera et al., 2010).

It has been shown that injecting oligodeoxyribonucleotides with CpG motifs (CpG ODN) stimulates immune parameters that can provide protection against diseases (Carrington & Secombes 2007; Martinez-Alonso et al., 2011; Nakatani et al., 2007; Pridgeon et al., 2012). CpG-ODN was encapsulated in PLGA/polysomal nanoparticles to be administered intraperitoneally to kelp groupers twice in a period of a week. PLGA, liposomes, and ODN PLGA/liposomes were able to stimulate RB, superoxide dismutase (SOD), complement and antibodies, the PLGA/liposome mixture causing the greatest effect. These intraperitoneal vaccinations decreased the mortality rate by *Vibrio alginolyticus* from 90% to 20, 15 and 15%, respectively. Injection with naked ODN decreased mortality caused by challenge by 20%, although there were lesser increases in SOD, RB and antibodies. The administration of PLGA, liposomes and PLGA/liposomes also decreased mortality by 30, 30 and 25% (Harikrishnan et al., 2012).

Viral antigens encapsulated in PLGA

Adomako et al. (2010) found that the administration of PLGA particles that encapsulate DNA coding for the G protein of IHNV by intramuscular injection or in feed mixed with soya derivatives caused the expression of the G protein in 13% of the vaccinated fish and stimulated antibodies in 15% of orally vaccinated fish and 27% of those vaccinated by intramuscular injection vaccination also protected a low percentage of rainbow trout against IHNV at 6 weeks of challenge, but this percentage was higher than that protected by naked DNA administered by IM. The authors note that the release of DNA from nanoparticles is too low at 14 °C to explain the low levels of transfection. However, they note that at pH 4; 7.7 and 8.4 a large amount of DNA was released in the first hour of incubation (15% at pH 4 and 20-25% at pH 7.7 and 8.4). This indicates that while the vaccine can be incorporated into different organs of the fish, this is not enough for an efficient expression of the DNA and that for DNA vaccines that are administered orally, the PLGA particles should be stabilized so that they do not degrade in the pH of the stomach and instead are incorporated by the cells in the intestine and can release the DNA in the cell nucleus to allow its expression (Adomako et al., 2012).

The PLGA nanoparticles of two strains of IPNV inactivated in formalin and administered to Atlantic salmon intraperitoneally stimulates antibodies to a comparable level to that provoked by inactivated virus administered in oily adjuvant or the recombinant VP2 protein. Nevertheless, the level of protection against IPNV is lower (Munang›andu et al., 2012).

The oral administration through filling-stomach syringes of PLGA of DNA encoding for a LCDV protein allows for the detection of a low quantity of 500-nm nanoparticles in the blood at two hours, with an increase at 8 hours (Tian & Yu 2011) and allows for the protein to be detected in gills, intestine, spleen and kidneys up to 90 days (Tian et al., 2008; Tian & Yu 2011). The nanoparticle vaccine stimulated the immune response, causing a significant increase in the number of antibodies and reaching a maximum at six to nine weeks post-vaccination (Tian & Yu 2011) compared to naked DNA with microparticles of approximately 1μm (Tian et al., 2008). The vaccination can induce a significant response in respiratory and proPO activity, suggesting an increase in phagocytosis and levels of SOD, lysozymes and antibodies also increase. This vaccine provides marked protection to Japanese flounders against the production of tumors. According to the Pearson correlation, only the antibodies in serum

did not correlate with the rest of the immune parameters obtained, indicating that this increase is not relevant to immunostimulation (Tian & Yu 2011).

Parasitic antigens encapsulated in PLGA

In the only study on parasites, the i-antigen (i-AG) of *Uronema marinum* was obtained by disrupting a expressing bacterin of, which was encapsulated in PLGA nanoparticles and administered intraperitoneally to Kelp grouper (*Epinephelus bruneus*). The administration of the nanoparticles increased the levels of lysozyme, RB, antiprotease, serum b2-macroglobulin and complement at post-vaccination weeks 1, 2 and 4. Antibodies increased to levels similar to when I-AG is injected naked. This vaccination succeeded in providing protection against infection, reaching an RPS of 20%, while the antigen without encapsulation and the empty PLGA nanoparticles reached RPS of 30 and 40% respectively (Harikrishnan et al., 2012).

This result is promising in the use of PLGA particles in encapsulating antigens against parasitic diseases such as *Caligus rogercresseyi,* also known as *Lepeophtheirus salmonis*, which has caused major losses in the aquaculture industry worldwide.

DISCUSSION

Research into generating particles to encapsulate molecules to be delivered to aquaculture species is in its initial stages. Published results show a great variation in efficacy depending on the method of administering the vaccine, the fish species and the method of encapsulation, among other factors. Unfortunately, the techniques employed in encapsulation are not often detailed. Likewise, there is often no characterization of the particles in terms of size, shape and physical-chemical properties, which makes it difficult to deduce what characteristics a particle should have to be efficient in the delivery of drugs to fish species. In general, the methods used are extrapolation to fish of the same strategies used with mammals, which could explain the low efficiency of these strategies. The environments in which mammals and fish live are different, as are the immune responses, the target cells, the tissue and organs and the pathogens that infect them.

In general, the administration of particles by IP has permitted the development of protection against the pathogen, while the oral administration of these vaccines offers low levels of protection, although with notable exceptions (De Las Heras et al., 2010; Tian & Yu 2011; Tian et al., 2008). Good results have also been obtained evaluating the use of antigen-encapsulating particles as a method for oral administration of boosters (Altun et al., 2010; Romalde et al., 2004), which offers a good non-invasive alternative for vaccinating fish. These results indicate that it is possible to develop encapsulating particles for oral administration, although improvements in their design are required.

As with evaluations of other fish vaccines, experiments with nanoparticle and microparticle vaccines show that the levels of neutralizing antibodies do not correlate with the degree of protection provided by the vaccine (Cuesta et al., 2010; De Las Heras et al., 2010; Dorson et al., 1978; Koellner & Kotterba 2002). Even when the number of antibodies increases, the Pearson correlation shows that the increase is not related to the protection against LCDV (Tian & Yu 2011). Notably, these antibodies are generally detected in serum and do not determine the effect of vaccination on the mucosal antibodies. The mucosal immunity in fish has not been well studied. It would be important to determine if the mucosal response correlates with the level of protection in order to develop methods of delivering vaccines that can activate mucosal antibodies. This would be highly compatible with the development of oral or immersion-based vaccines that allow for the primary action of the antigen with the mucous of the intestine, stomach and gills. Consequently, it is also important to keep in mind that the choice of method of inoculation can depend on the type of pathogen and the target organ that is infected. Oral vaccines accumulate in the intestine, injected vaccines in the abdominal cavity or in the muscles, and vaccines by immersion accumulate in the gills. In this respect, the choice of cells to deliver the vaccine, such as APC or target cells of the pathogen is limited by lack of knowledge about the characteristics of fish cells, which differ in several respects from mammalian cells. Among the differences are slower growth at lower temperatures of fish cells, less sensitivity to toxic compounds, and different compositions of membranes and proteins of different amino acid sequences that carry out the same functions in the cells of fish and that are not immunogenic in that they can generate antibodies to detect them. There are cell types in fish that have not been clearly identified in mammals, such as nucleated erythrocytes (Glomski et al., 1992), phagocyted lymphocytes B (Li et al., 2006) and finally organs that bring together the functions of several organs in mammals or functions in fish that are different from those in mammals. It is necessary to understand these differences in fish cells in order to design vaccination methods for efficient delivery to the organs and cells of the fish.

Added to the above, different fish species have different responses to vaccination (Joosten et al., 1997), so that the methods of encapsulation may not be transferable from one species to another. As well, encapsulated antigens modify the physical-chemical characteristics of the particle so that the results of assays on stability, physical-chemical parameters and the location in organs cannot be extrapolated from one molecule to another using the same encapsulating particle. Similarly, the characteristics of the antigen can be changed when it is encapsulated, because of which the function, structure, stability and immunogenicity of the antigen needs to be maintained and verified.

It has been demonstrated that the use of encapsulating DNA particles that encodes for pathogenic proteins provides a high level of protection in fish against pathogens when alginate, chitosan and PLGA are used. Results have been very promising given that the inoculation of DNA has been shown to be very effective with fish. In fact, antigen-coding naked DNA has been highly effective against a variety of viral strains of rhabdovirus among different fish species in different states of development (Kim et al., 2000; Lapatra et al., 2001; Lorenzen et al., 2002), leading to the development of a commercial prototype against IHNV by Aqua Health Ltd in Canada, which is the only country that has allowed the use of DNA vaccines in fish farming. The advantages of DNA vaccines are their safety, it being impossible that they cause infection. They avoid the synthesis of antigenic protein, trigger long-term immune and humoral response, are effective in early stages of growth,

and are inexpensive and easily stored. The administration of DNA in fish stimulates the expression of MHC class II and the presence of unmethylated CpG activates macrophages, causes the proliferation of leukocytes and stimulates the expression of cytokines (Lee et al., 2003; Tassakka & Sakai 2004; Boudinot et al., 1998; Meng et al., 2003; Tassakka & Sakai 2003), acting as adjuvants (Kanellos et al., 1999; Martinez-Alonso et al., 2011). The disadvantage of these vaccines is that naked DNA must be administered by intramuscular injection (Salgado-Miranda et al., 2013) or by particle-mediated delivery by gene guns (Corbeil et al., 2000; Gomez-Chiarri et al., 1996; Sudha et al., 2001), given that oral administration causes degradation of the DNA due to the low pH level in the stomach and the DNases in the gastrointestinal tract. Consequently, adequate DNA encapsulation would be the ideal way to administer these vaccines orally.

The current use of particles to deliver antivirals or antibiotics in viral or bacterial target cells or of immunostimulants that can maintain a state of immunity in fish has been reported. These can be co-administered with vaccines to strengthen them.

Finally, legislation is needed that allows for the use of particles to administer drugs to fish orally or by immersion. The safety of the vaccines needs to be studied, given that the vaccination material can escape to the environment from immersion pools or corrals during vaccination, making it possible that there will be human contact with the vaccine. As well, there need to be studies of the adverse effects on fish. A correct protocol based on knowledge of adverse effects can result in appropriate use of encapsulating particles in fish farming.

All of the above indicates that antigen encapsulation of fish vaccines is very promising, although more research is needed to develop specific encapsulation methods for fish vaccines that stimulate adequate immune responses.

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