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Recent Advances in Application of Nanoparticles in Fish Vaccine Delivery

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ABSTRACT

There is a constant need for the development of efficient vaccines and delivery systems to prevent and control the emerging and re-emerging infectious diseases in aquaculture. There are innumerable infectious diseases for which the development of efficient vaccines has been difficult to achieve. The failure is mainly due to the inability to design vaccines evoking appropriate immune responses. The use of nanoparticles has provided a tremendous opportunity to design vaccine delivery systems that are efficient in targeted delivery, providing stability to antigens, and act as efficient adjuvants. Many of the nanoparticles are able to enter the antigen presenting cells by different pathways and induce appropriate immune responses to the antigen. A number of different nanoparticles are used in fish vaccine delivery, which includes biodegradable polymers, nanoliposomes, carbon nanotubes, calcium phosphate, and immunostimulating complexes (ISCOMs), among which poly (lactic-co-glycolic acid) and chitosan are the most studied form of nanoparticles to date. Hence, the use and application of other forms of nanoparticles need to be explored. This review provides an overview of the use of different nanoparticle systems for the delivery of fish vaccines and compares the potential of these delivery systems for the development of new vaccines against different fish pathogens.

KEYWORDS

Nanovaccine; nanoparticles; vaccine delivery; adjuvant; fish vaccine; nanotoxicity

Introduction

Aquaculture production has expanded from being negligible to fully comparable with capture productions and has become the fastest growing food producing sectors. The global aquaculture production has reached 73.8 million tons in 2014 and it is increasing steadily (FAO, 2016). The increased human population has an ever increasing demand for fish and the aquaculture systems are getting intensified to meet the demand. Intensification comes with the risk of increased disease outbreaks from emerging and re-emerging pathogens. By using different prophylactic measures, the area of interest for managing fatal disease outbreaks in sustainable intensified-aquaculture systems can be met out. In this regard, development and application of vaccines play a major role (Brudeseth et al., 2013).

Vaccination has had a major impact on control and prevention of infectious diseases in aquaculture (Brudeseth et al., 2013; Carmen and Forlenza, 2016) despite that there are many infectious diseases for which the development of an effective vaccine has been difficult to achieve. Traditionally as explained by Louis Pasteur, it is based on the principle of isolate, inactivate, and inject (Zhao et al., 2014). The vaccine development has had a transition from this conventional method of using whole pathogen to using only the required protein and peptide antigens that have reduced the unwanted side effects but the immunogenicity of these antigens has gone down drastically (Smith et al., 2015). To enhance the immunogenicity of vaccines use of adjuvants and efficient delivery systems is very essential (Petrovsky and Aguliar, 2004; Corradin and Giudice, 2005; Evensen et al., 2005; Evensen, 2009). Adjuvants help to enhance the immune response and also to reduce the frequency of administration (Evensen, 2009). Mineral oil is the most commonly used adjuvant but most of the vaccines containing mineral oil as adjuvant cause serious side effects that include granulomas, adhesion, pigmentation, poor feeding, and growth retardation (Midtlyng et al., 1996; Poppe and Breck, 1997; Midtlyng and Lillehaug, 1998; Bowden et al., 2003; Mutoloki et al., 2004; Evensen et al., 2005;

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Drangsholt et al., 2011). Hence, it is necessary to develop novel adjuvants and delivery systems that are safe and potent for aquacultured species.

Recent research has been focusing on the use of nanoparticles (NPs) as adjuvants and efficient delivery systems in fish vaccine development. Nanoparticles are known to exhibit interesting properties that are different from their parent materials, which include: increased relative surface area and quantum size effects. These characteristics of nanoparticles are of great importance in terms of application in medical field (Yildirimir et al., 2011). Thus, nanotechnology is the ability to manipulate these tiny particles (Jaradat, 2013; Cavalieri et al., 2014; Shaalan et al., 2016), varying in size, shape, composition, and surface properties (Oberdorster et al., 2005; Zhao et al., 2014; Shaalan et al., 2016). Due to their nanosize, nanoparticles can be taken up by cellular endocytosis mechanism (Zaman et al., 2013; Zhao et al., 2014), which facilitate the cellular uptake of antigens and increase the ability of antigen presentation (Oyewumi et al., 2010; Kim et al., 2014; Shaalan et al., 2016). Studies have demonstrated that application of nanotechnology increases the solubility, stability, targeting, biocompatibility, and permeability of vaccines (Frohlich, 2012; You et al., 2012; Doll et al., 2013; Lai et al., 2013). Nanotechnology converging with biotechnology has made a significant progress in biomedicine (Pankhurst et al., 2003; Tissot et al., 2008; Zhao et al., 2014) and its application has increased in the field of vaccinology giving rise to a new field of science called "nanovaccinology" (Mamo and Poland 2012; Zhao et al., 2014). Nanovaccines, thus developed are made up of nanoparticles formulated with antigens either encapsulated within or adsorbed on to the surface against which an immune response is desired (Gregory et al., 2013; Zaman et al., 2013). A schematic illustration of nanovaccine development and evaluation is shown in Figure 1. The advantages of nanovaccines include: protection of antigens by encapsulation from degradation, site specific delivery of antigens, enhanced bioavailability, and reduced side effects (Zolnik et al., 2010; Gregory et al., 2013; Zaman et al., 2013).

This review presents an overview of various nanoparticle-based fish vaccines with an expectation that the nanovaccines developed using this technology will be better than the conventional methods in providing antigen-specific immune response, which may encourage to devise vaccines for those infectious diseases against which the development of effective vaccines has been difficult.

Principles of innate and adaptive immunity in fish

The main goal of vaccination is to obtain protection and immunity against pathogens by triggering the immune system (Pulendran and Ahmed, 2011; Brudeseth et al., 2013; Sahdev et al., 2014). Fish immune system is broadly classified into innate and adaptive components. The innate defense mechanism in fish is activated quickly upon infection and it includes surface barriers (mucus, skin, gills, gastrointestinal tract), growth inhibitors (transferrin, interferon), enzyme inhibitors, lysins (complement, antimicrobial peptides, lysozyme), precipitins and agglutinins (pentraxins, lectins), nonspecific cellular factors like phagocytes (macrophages, neutrophils), phagocyte activating molecules (opsonins, cytokines), natural cytotoxic

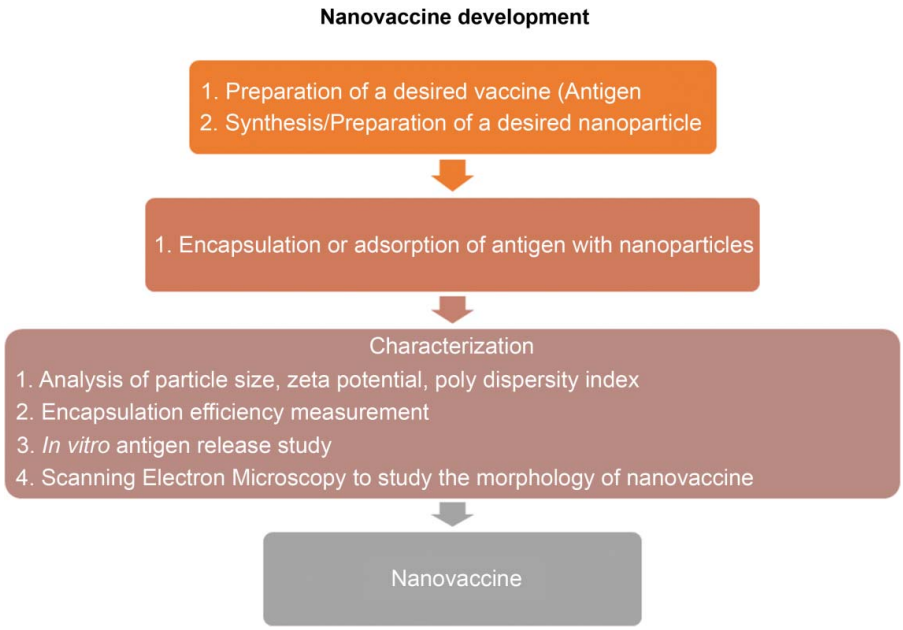

Figure 1. Schematic illustration of a nanovaccine development.

cells, eosinophils, basophils, mast cells, and inflammation (Magnadottir, 2006; Secombes and Ellis, 2012). On the other hand, adaptive immune responses take several days to become effective, but provide specific memory, which is required for complete elimination of the pathogen (Tort et al., 2003; Secombes and Ellis, 2012). There are three aspects of adaptive immune system mediated by lymphocyte: *humoral immunity, cell-mediated immunity*, and *immunological memory*. Humoral immunity is marked by the production of immunoglobulins (Ig) produced by B-cells, and in fish there are three types of Ig's known till date (IgM, IgD, and IgT; Hansen et al., 2005; Hu et al., 2010; Ballesteros et al., 2013). Nanoparticle-based vaccines have been successful in mounting specific immune response against the antigen. Several studies have reported the generation of specific antibodies even against nanoparticles, which is not a desired attribute as it may affect the efficacy of nanovaccines (Chen et al., 1998; Braden et al., 2000; Lee et al., 2004; Zolnik et al., 2010; Zaman et al., 2013). In general, nanoparticles are not antigenic by themselves but they show antigenic properties when conjugated with antigens (proteins) due to the increased size of the particles (Zolnik et al., 2010; Zaman et al., 2013). Cell-mediated immunity is a function of T-cells to combat the infections and the cellular components and is essential to provide protection against many pathogens. Several studies in non-fish model have demonstrated the induction of robust cellular immune response upon administration of nanoparticle-based vaccines (Demento et al., 2010; Gregory et al., 2013; Zaman et al., 2013). The immunological memory is an important aspect in specific immune response, which comprises the adaptive change in lymphoid cells, so that the next time when fish are exposed to that particular pathogen, the immune system recognizes it immediately and destroys it before it can do serious damage and this is the basis for successful vaccination approach (Secombes and Ellis, 2012). The development of a vaccine includes both the triggering of innate and adaptive immune systems, which actually works in a coordinated manner. Further, to enhance and prolong the immune response, the use of adjuvants and delivery system becomes inevitable and various nanoparticles have emerged as frontrunners due to their unique properties. Nanoparticle-based vaccines help to bridge the gap by inducing the up-regulation of several inflammatory, innate, and specific immune responsive genes (Zhu et al., 2014, 2015; Wang et al., 2015; Zheng et al., 2016).

Nanoparticles as adjuvants and delivery systems

Formulation of vaccines with nanoparticles has opened up tremendous opportunities in the field of biomedicine (Cavalieri et al., 2014; Shaalan et al., 2016). Nanoparticles in vaccine development can be grouped according to their action, either as an efficient mode of delivery system or an adjuvant. Nanoparticles that function as delivery systems will deliver the antigen to targeted immune cells while protecting it and immune potentiating adjuvant nanoparticles will activate specific pathways, which helps in efficient antigen uptake and processing (Hølvold et al., 2013; Tafalla et al., 2013; Zhao et al., 2014,). Further, the nanoparticles can be classified as biodegradable or non-biodegradable based on their properties of decomposition in biological system. The widely investigated nanoparticles are biodegradable polymers to harness the advantages like the controlled release of antigen, gastrointestinal stability, and safety (Myhr and Myskja, 2011; Zhao et al., 2014; Shaalan et al., 2016). In general, the other forms of nanoparticles used in vaccine studies include: virus-like particles (VPLs), nanoliposomes, immunostimulating complexes (ISCOMs), nanoemulsions, and metal nanoparticles (Gregory et al., 2013; Zhao et al., 2014; Shaalan et al., 2016). Table 1 provides the details of types of nanoparticles applied in vaccine research with their merits and demerits.

Table 1. Merits and demerits of nanoparticles.

Type of nanoparticles	Merits	Demerits
Polymeric nanoparticles	Better immunogenicity can be obtained by easy modification of surface properties, biodegradable and targeted antigen delivery	Low aqueous solubility and synthesis requires use of organic solvents, low antigen loading, premature release of antigens, insufficient antigen protection
Inorganic nanoparticles	Easy to modify, less chances of premature release, and better protection of adsorbed antigens	Low aqueous solubility and low biodegradability
Nanoliposomes	Possess intrinsic adjuvant properties, accommodates both hydrophilic and lipophilic antigens, and relatively stable in gastrointestinal fluids when modified	Low mucus penetration, limited antigen loading, and poor gastrointestinal stability of naked liposomes
ISCOMS	Easy to encapsulate and built in adjuvant property of Quil A	Do not form depot and difficult to incorporate hydrophilic antigens
Virus like particles	Possess self-adjuvant properties, mimics original virus and high gastrointestinal stability	Lack of reproducibility
Nanoemulsions	Possess self-adjuvant properties, encapsulates both hydrophilic and lipophilic antigens	Premature release of antigens and poor gastrointestinal stability

The type of nanoparticles used in developing fish vaccines is restricted mainly to polymeric nanoparticles, nanoliposomes, carbon nanotubes, calcium phosphate, ISCOMs, and the application of other forms of nanoparticles need to be explored. Though there is an obvious advantage in development of nanovaccines, there are few concerns mainly in maintaining the stability and consistency of nanoparticle properties and their toxicity (Lai et al., 2013), which needs to be addressed carefully. This article will focus on the potential and application of inorganic, polymeric, and biomolecular nanoparticles, which have been applied in developing efficient fish vaccines. The present status of nanoparticle-based fish vaccines is summarized in Table 2.

Inorganic nanoparticles

Inorganic nanoparticles are used in vaccine research both as adjuvants and potential vaccine delivery systems due to their attractive physical and chemical properties (Sahdev et al., 2014; Zhao et al., 2014). Structurally they are solid particles, which can be conjugated with antigen and it provides better gastrointestinal and storage stability with higher antigen encapsulation efficiency and targeted delivery (Smith et al., 2015). There are several inorganic nanoparticles based on carbon, calcium phosphate, gold, silver, silicate, aluminium, titanium, etc., among which carbon nanotubes and calcium phosphate are evaluated as vaccine delivery systems in fish vaccines.

Carbon nanotubes (CNTs) can be listed as emerging nanoparticles in the biomedical research and are investigated as antigen delivery systems (Kim et al., 2014; Ji et al., 2015). There are two main types; single-walled and multi-walled nanotubes that are insoluble and non-degradable (Scheinberg et al., 2013; Kim et al., 2014). Carbon nanotubes are very stable, lack intrinsic immunogenicity, and are capable of carrying multiple antigens, which qualifies them for potential vaccine delivery particles. The application of CNTs in fish vaccine delivery is quite recent and has demonstrated the efficacy both in intramuscular injection route as well as in immersion treatments of DNA vaccines produced against VP5 and VP7 genes of grass carp reovirus (GCRV) in a dose-dependent manner. The DNA vaccine without CNTs was less protective and the presence of CNTs in vaccine formulation clearly up-regulated several inflammatory, innate, and adaptive immune genes helping to mount a strong immune response against the pathogen (Zhu et al., 2014, 2015; Wang et al., 2015).

Calcium phosphate is a naturally occurring inorganic compound in our body. It can easily be formed by mixing calcium chloride, sodium citrate, and sodium phosphate. Since it is present in the body, it is biocompatible and non-toxic and easy to manufacture. It has a very high affinity to proteins, hence, making it one of the suitable candidate nanoparticle for vaccine delivery (Dorozhkin and Epple, 2002; Sokolova et al., 2006; Boutinguiza et al., 2011; Behera and Swain, 2011; Smith et al., 2015). An interesting study has demonstrated the use of calcium phosphate adsorbed to S-layer protein of *A. hydrophila* stimulating both innate and adaptive immune response of fish providing complete protection against the infection (Behera and Swain, 2011).

The inorganic nanoparticles have good adjuvant properties and stabilities but they have certain limitations in their chemistry and physical properties. Due to their varied chemistry, polymeric nanoparticles are the widely used nanoparticles in vaccine research. The most explored nanoparticles in fish vaccine studies are the polymeric PLGA and chitosan for administration of viral as well as bacterial antigens.

Polymeric nanoparticles

The most preferred nanoparticles in vaccine research are the polymeric nanoparticles due to their biodegradable nature, biocompatibility, and diverse chemical properties. Polymeric nanoparticles have the capacity to conjugate or encapsulate any antigens within itself or on their surface (Marasini et al., 2014; Sahdev et al., 2014). There are several polymeric nanoparticles that can be grouped based on their origin as: Naturally derived and synthetically derived polymers.

Naturally derived polymers

Chitosan is a naturally derived biodegradable polymer and is extracted from various chitinous materials mainly from the exoskeleton of crustaceans and hence it can be earmarked as a green nanoparticle. It is highly abundant, biodegradable, and biocompatible, making it an attractive candidate for vaccine delivery (Sahdev et al., 2014). Chitosan nanoparticles can be formed by ionotropic gelation and self-assembly of polyelectrolytes, which help in retaining immunogenicity of the encapsulated antigens (Arca et al., 2009; Sahdev et al., 2014; Younes and Rinaudo, 2015). It has the ability to stimulate good adaptive immune response, both cellular and humoral against the conjugated antigen (Arca et al., 2009). The use of chitosan nanoparticles in fish vaccines has a great advantage as it can be used to enhance the mucosal immunity through oral route of vaccination in fish (Carmen and Forlenza, 2016). Several oral DNA vaccination studies have demonstrated the increased effectiveness of chitosan nanoparticles in comparison to other formulations in fish against the antigens derived from

Table 2. Experimental approaches in nanoparticle based fish vaccine delivery.

Nanoparticle[a]	Antigen	Pathogen[b]	Species	Vaccine route[c]	Vaccine formulations with nanoparticles	RPS[d]	Reference
Chitosan	rgpG	VHSV	*Danio rerio*	IP	NPrgpG	70	Kavaliauskis et al. (2016)
					plCrgpG	55	
					CSrgpG	0	
					NpiV	81	
Chitosan	pEGFP-N2-TRBIV-MCP (pDNA)	TRBIV	*Scophthalmus maximus*	Oral	pDNA-CS-NPs	68.2	Zheng et al. (2016)
Chitosan	ISAV (V)	ISAV	*Salmo salar*	Oral	NP-V	40.4	Aravena et al. (2015)
					NP-Ad+NP-V	77.9	
Chitosan/TPP	pFNCPE42	Nodavirus	*Lates calcarifer*	Oral	pFNCPE42-CS/TPP	60	Vimal et al. (2014)
Chitosan	OmpK	*Vibrio parahaemolyticus*	*Acanthopagrus schlegelii*	Oral	pEGFP-N2-OMPK	72.3	Li et al. (2013)
Chitosan/TPP	pVAOMP-DNA	*Vibrio anguillarum*	*Lates calcarifer*	Oral	CS/TPP- pVAOMP-DNA	ND	Vimal et al. (2012)
Chitosan	pVAOMP38	*Vibrio anguillarum (Listonella)*	*Lates calcarifer*	Oral	Chitosan-pVAOMP38	46	Rajesh et al. (2008)
OCMCS-hyaluronic acid	aerA	*Aeromonas hydrophila*	*Cyprinus carpio*	Oral	OCMCS/aerA-NPs	ND	Liu et al. (2016)
					OCMCS-HA/aerA-NPs		
Alginate	Irradiated trophont *(Radiovac)*	*I. multifiliis*	*Oncorhynchus mykiss*	Oral	NP-gamma-irradiated trophont	—	Heidarieh et al. (2015)
PLGA	rOmpW	*Aeromonas hydrophila*	*Labeo rohita*	Oral	Np-rOmpW (HiAg) 8 μg/g	79.9	Dubey et al. (2016)
					Np-rOmpW (LoAg) 4 μg/g	37.3	
					NPs	3.9	
PMMMA-PLGA	SIP	*Streptococcus agalactiae*	*Oreochromis niloticus*	Oral	PTRBL/Trx-SIP	100	Zhang et al. (2015)
PLGA,PLA	Omp	*Aeromonas hydrophila*	*Labeo rohita*	IP	PLGA-Omp	75	Rauta and Nayak (2015)
					PLA-Omp	80	
PLGA	TA,PT	IPNV	*Salmo salar*	IP	PLGA nanoparticle-TA	16.7	Munang'andu et al. (2012)
					PLGA nanoparticle-PT	21.1	
PLGA	pCDNA-G	IHNV	*Oncorhynchus mykiss*	Oral	(6 WPV)		Adomako et al. (2012)
					Low dose PLGA-pCDNA-G	11	
					High dose PLGA-pCDNA-G	22	
					High dose PLGA	6	
					(10WPV)	0	
					Low dose PLGA-pCDNA-G	19	
					High dose PLGA-pCDNA-G	16	
					High dose PLGA		
PLGA,PLA	HGG	—	*Salmo salar*	IP	PLGA-NP50L	ND	Fredriksen and Grip (2012)
					PLGA-NP50H		
					PLGA-NP75H		
					PLA-NP100L		
PLGA	pEGFP-N2-MCP	LCDV	*Paralichthys olivaceus*	Oral	pEGFP-N2-MCP	ND	Tian and Yu (2011)
					PLGA		
					PLGA- pEGFP-N2-MCP		
PLGA	TNP-LPH	—	*Salmo salar*	IP	NP	ND	Fredriksen et al. (2011)
					NP/TNP-LPH		
					NP/β glucan		
					NP/TNP-LPH /β glucan		

(*Continued on next page*)

Table 2. (Continued).

Nanoparticle[a]	Antigen	Pathogen[b]	Species	Vaccine route[c]	Vaccine formulations with nanoparticles	RPS[d]	Reference
Liposome	*Vibrio harveyi*	*Vibrio harveyi*	*Epinephelus bruneus*	IP	Liposome-*V. harveyi*	75	Harikrishnan et al. (2012)
Liposome	NKC03,IKC03	KHV	*Cyprinus carpio*	Oral	Liposome-NKC03	74.3	Yasumoto et al. (2006)
					Liposome-NKC03	65	
Liposome	*Aeromonas salmonicida (T1031)*	*Aeromonas salmonicida*	*Cyprinus carpio*	Oral	Liposome-T1031	83.3	Irie et al. (2005)
Carbon nanotubes	rVP7	GCRV	*Ctenopharyngon idellus*	Bath	(0.2 g-Fish)	37.7	Zhu et al. (2014)
				IM	SWCNTs- vp7 2.5 mg/L	52.3	
				Bath	SWCNTs -vp7 5 mg/L	86.8	
				IM	SWCNTs- vp7 10 mg/L	94.6	
					SWCNTs- vp7 20 mg/L	97.4	
					SWCNTs- vp7 40 mg/L	8.6	
					SWCNTs- vp7 0.2 μg	28.7	
					SWCNTs- vp7 0.4 μg	44.3	
					SWCNTs- vp7 0.6 μg	85.8	
					SWCNTs- vp7 0.8 μg	93.1	
					SWCNTs- vp7 1.0 μg	33	
					(25 g-Fish)	48.5	
					SWCNTs- vp7 2.5 mg/L	83.7	
					SWCNTs -vp7 5 mg/L	89.9	
					SWCNTs- vp7 10 mg/L	95.6	
					SWCNTs- vp7 10 mg/L	7.1	
					SWCNTs- vp7 40 mg/L	24.2	
					SWCNTs- vp7 0.2 μg	41.4	
					SWCNTs- vp7 0.4 μg	87.7	
					SWCNTs- vp7 0.6 μg	93.2	
					SWCNTs- vp7 0.8 μg		
					SWCNTs- vp7 1.0 μg		
Carbon nanotubes	pEGFP-vp5	GCRV	*Ctenopharyngon idellus*	IM	SWCNTs- pEGFP-vp5 1 μg	56.7	Wang et al. (2015)
				Bath	SWCNTs- pEGFP-vp5 2.5 μg	90	
					SWCNTs- pEGFP-vp5 5 μg	100	
					SWCNTs- pEGFP-vp5 1 mg/L	40	
					SWCNTs- pEGFP-vp5 10 mg/L	63.3	
					SWCNTs- pEGFP-vp5 20 mg/L	96.7	
Carbon nanotubes	pcDNA-vp7	GCRV	*Ctenopharyngon idellus*	IM	SWCNTs- pcDNA-vp7 1 μg	72.5	Zhu et al. (2015)
					SWCNTs- pcDNA-vp7 5 μg	90.8	
					SWCNTs- pcDNA-vp7 10 μg	100	
Calcium phosphate	S-layer protein	*Aeromonas hydrophila*	*Labeo rohita*	IP	SP-CaNP	100	Behera and Swain (2011)
ISCOMs	MOMP	*Aeromonas hydrophila*	*Anguillia anguillia*	IP	MOMP-ISCOMs	80	Dong et al. (2005)

[a]PLGA: Poly (Lactic-Co-Glycolic Acid), OCMCS: Oleoyl-carboxymethyl-chitosan, PMMMA: Poly [(methyl methacrylate)-co-(methylacrylate)-co- (methacrylic acid)], PLA: Poly (Lactic- Acid), ISCOMs: Immunostimulating Complexes.
[b]VHSV: Viral hemorrhagic septecemia virus. TRBIV: Turbot reddish body iridovirus, GCRC: Grass carp reovirus, ISAV: Infectious salmon anemia virus, IPNV: Infectious pancreatic necrosis virus, IHNV: Infectious hematopoietic necrosis virus, LCDV: Lymphocystis disease virus, KHV: Koi herpes virus.
[c]IM: Intramuscular, IP: Intraperitoneal.
[d]RPS: Relative Percentage Survival, ND: Not determined.

turbot reddish body iridovirus (TRBIV), Nodavirus, *Vibrio parahaemolyticus, Vibrio anguillarum* (Rajesh et al., 2008; Vimal et al., 2012, 2014; Li et al., 2013; Zheng et al., 2016). Aravena et al. (2015) have also demonstrated the effectiveness of chitosan nanoformulations even against the inactivated infectious salmon anemia virus (ISAV). A recent study has reported the efficacy of chitosan in intraperitoneal administration of vaccine against viral hemorrhagic septecemia (VHSV) recombinant glycoprotein (rgpG) in zebrafish model (Kavaliauskis et al., 2016). The presence of chitosan nanoparticles along with another adjuvant poly (I:C) showed enhanced protective response in comparison to other formulations. Collectively, the research suggests that the use of chitosan nanoparticles enhances the vaccine-mediated protection against the infection in fish.

Hyaluronic acid (HA) is a natural polymer composed of D-glucuronic acid and N-Acetyl-D-glucosamine and is a component of cartilaginous tissue (Sahdev et al., 2014; Smith et al., 2015). It also plays an important role in immune response by modulating leukocyte trafficking (Mummert, 2005; Sahdev et al., 2014). It is biocompatible, biodegradable, hydrophilic and due to high abundance in nature and makes it as one of the attractive candidate nanoparticle for vaccine delivery (Sahdev et al., 2014; Smith et al., 2015). Liu et al. (2016) demonstrated the use of nano-polyplexes made of oleoyl-carboxymethyl-chitosan in conjugation with HA to form a more physiologically stable DNA vaccine (aerolysin gene of *Aeromonas hydrophila*) carrier in fish.

Alginate is an extract of naturally available brown algae and is found as a polysaccharide in some bacteria. It is made of repeated units of unbranched polyanionic polysaccharides α-L-guluronic acid and β-D-mannuronic acid (Ji et al., 2015). It is biodegradable, biocompatible, non-toxic, acid resistant, mucoadhesive and most suited for oral vaccine delivery (Wee and Gombotz, 1998; Aravena et al., 2013). Alginate has been used in fish vaccine delivery in the form of microparticle formulations more often than nanoformulations, and there has been a recent report that evaluated alginate nanoparticles for oral vaccine delivery against *Ichthyophytirius multifiliis* in rainbow trout for booster vaccination (Heidarieh et al., 2015).

Synthetically derived polymers

Poly (lactic-co-glycolic acid; PLGA) is a synthetic copolymer of lactic acid and poly glycolic acid. It is a very commonly used delivery system in biomedical research. The use of PLGA is approved by US-FDA and European Medicine Agency (EMA) due to its biocompatibility, non-toxicity, and highly biodegradable nature. Upon administration it undergoes hydrolysis and release glycolic and lactic acids that are eventually removed from body by citric acid cycle (Panyam and Labhasetwar, 2003; Sahdev et al., 2014; Ji et al., 2015). Poly (lactic-co-glycolic acid) is also used as an adjuvant, alternative to alum for prolonging the in vivo antigenic exposure time (Toita et al., 2013; Smith et al., 2015). It is generally used for the controlled release of nucleic acids, proteins, and peptides and hence it is the most explored nanoparticle for the delivery of fish vaccines (Fredriksen et al., 2011; Tian and Yu, 2011; Adomako et al., 2012; Fredriksen and Grip, 2012; Munang'andu et al., 2012; Rauta and Nayak, 2015; Zhang et al., 2015; Dubey et al., 2016). As it is stable in gastric conditions, it has been used for the efficient oral delivery of recombinant outer membrane protein W (rOmpW) of *A. hydrophila*, surface immunogenic protein (SIP) of *Streptococcus agalactiae*, DNA vaccines against the G gene of infectious hematopoietic necrosis virus (IHNV), and lymphocystis disease virus (LCDV; Tian and Yu, 2011; Adomako et al., 2012; Zhang et al., 2015; Dubey et al., 2016). PLGA-based formulations with the G gene of IHNV were able to induce immune response through oral delivery, but the results obtained are inferior to intramuscular vaccination of DNA vaccine alone. This suggests the need for improvement in oral formulations, which can be observed in the case of PLGA-based "intelligent shell," containing SIP of *S. agalactiae* providing complete protection (Zhang et al., 2015). Similarly, immune response against outer membrane protein (Omp) of *A. hydrophila* (Rauta and Nayak, 2015) obtained in comparison to a poor response elicited by inactivated virus-based vaccine against infectious pancreatic necrosis virus (IPNV) in intraperitoneal administration (Munang'andu et al., 2012).

Poly (lactic acid; PLA) is a synthetic polymer comprising of repeated lactide monomers that degrades into biocompatible lactic acid. It is less degradable compared to PLGA and hence has a limited usage as a vaccine delivery system (Smith et al., 2015). Only a single study is available in comparison to PLGA where PLA showed a better immune response against outer membrane protein (Omp) of *A. hydrophila* (Rauta and Nayak, 2015) without any statistical significance.

Lipid-based biomolecular nanoparticles

Biomolecular nanoparticles use bio-molecules as their base components. They are attractive materials to develop vaccine delivery systems as these can be designed as desired to carry the antigens (Kim et al., 2014). There are several biomolecular based nano-formulations used extensively in vaccine research such as liposomes, ISCOMs, micelles, and virus-like particles.

Among these, liposomes and ISCOMs are used for fish vaccine delivery (Kim et al., 2014). Nanoliposomes have been well documented for their diverse ability to deliver various hydrophilic and hydrophobic antigens as they possess hydrophilic head and hydrophobic tail (Kim et al., 2014; Ji et al., 2015; Smith et al., 2015). These are formed by non-toxic and biodegradable self-assembled structures of phospholipids consisting of an internal aqueous core entrapped by a lipid bilayer (Kim et al., 2014; Zhao et al., 2014). Surface modification of liposomes is easy and it can increase the immunogenicity to enhance both humoral and cell-mediated immunity (Kim et al., 2014). Few studies are reported in fish vaccine delivery using nanoliposomes, where fish were vaccinated orally with liposome nanoparticle-entrapped *A. salmonicida* and with koi herpes virus (KHV) providing efficient response over other formulations (Irie et al., 2005; Yasumoto et al., 2006).

There is a report on the efficacy of nanoliposome in intraperitoneal administration of vaccine against *Vibrio harveyi* (Harikrishnan et al., 2012).

Immunostimulating complexes (ISCOMs) are self-assembled cage-like structures usually of 40 nanometer size and consisting of cholesterols, phospholipids, and Quil A saponin. The cage-like structures help in entrapping the antigens or adjuvants. Immunostimulating complexes are good antigen carriers and are very efficient adjuvants as they are formed of saponin (Marasini et al., 2014; Zhao et al., 2014; Smith et al., 2015). Immunostimulating complexes are researched for more than three decades now and are restricted to veterinary use due to the mild toxic effects having hemolytic properties (Sjolander et al., 1998; Marasini et al., 2014; Smith et al., 2015). Though other forms of saponin are studied in fish vaccines as adjuvants, there is only a single study available in the nano form (ISCOMs) for vaccine delivery where, major outer membrane protein (MOMP) of *A. hydrophila* was entrapped in ISCOMs and delivered intraperitonially to eels, which provided good protection (Dong et al., 2005).

Nanoparticles and the associated immune responses

Nanoparticles induce different immune responses upon their administration without being immunogenic by themselves, unless they have been conjugated with an antigen. Different mechanisms are involved in the induction of immune responses by various nanoparticles, including pattern recognition receptors (PRRs) activation, cytotoxic T-lymphocyte induction, T-helper (Th) activation, cytokine production in diverse ways, B-cells activation, and antibody production (Najafi-Hajivar et al., 2016). Particle size is suggested to be a key factor in determining the type of immunity induced. Depending on their size, nanoparticles are taken up by antigen presenting cells (APCs) via different pathways, including both pinocytosis and phagocytosis (O'Hagan et al., 2001; Fifis et al., 2004). Several studies have reported that smaller particles elicit stronger immune responses than their larger counterparts (O'Hagan et al., 2001; Fifis et al., 2004; Minigo et al., 2007; Mottram et al., 2007; Manolova et al., 2008). Table 3 provides details on the type of immunity provided by various nanoparticles used in vaccine delivery.

Table 3. Type of immunity provided by various nanoparticles used in vaccine delivery.

Nanoparticle	Size of nanoparticle (average size in nanometers)	Type of immunity
Polymeric (PLGA)	~100–400 nm	Immunostimulation
Polymeric (Chitosan)	~200 nm	Immunostimulation
Inorganic (Carbon nanotubes)	~10–20 nm	Immunostimulation, Inflammation
Inorganic (Calcium phosphate)	~200 nm	Immunostimulation, Inflammation
Nanoliposomes	~100–400 nm	Hypersensitivity, Inflammation
Nanoemulsions	~50–600 nm	Immunostimulation/ modulation
ISCOMs	~40 nm	Immunostimulation, Inflammation
VLPs	~20–200 nm	Immunostimulation

Biosafety concerns of nanoparticle toxicity

While the nanoparticles have shown the undisputable potential for their wide range of applications, the very nature which make them interesting might have negative effects as well (Elsaesser and Howard, 2012; Gregory et al., 2013; Zellner, 2015). Since, they can cross the blood brain barrier (BBB), the applications have to be made carefully as it may cause serious problem (Yildirimir et al., 2011). The evaluation of nanoparticle toxicity is not easy and cannot be predicted based on the toxicity profile of their parent material as they exhibit different properties and are also taken up by cells in an entirely different way as compared to their parent materials. Recent study focused on understanding the mechanism of nanoparticle toxicity suggests that the toxicity may range from cell necrosis to reactive oxygen species (ROS) induced apoptosis (Elsaesser and Howard, 2012).

Nanoparticles used for the development of fish vaccines needs to be safe, biodegradable, or are able to be excreted from the fish's body as the fish is ultimately consumed by humans and there are chances of ingesting the nanoparticles remained un-degraded in fish. Most of the nanoparticles used in nanovaccine research are

biodegradable, hence safe for both fish and humans. For example, the polymeric nanoparticles like PLGA and chitosan used often for vaccine delivery in fish are safe even at very high concentrations and are also biodegradable (Yildirimir et al., 2011). The lipid-based nanoparticles are also safe to use without any toxicity issues (Nassimi et al., 2010), while the saponin content in ISCOM's may lead to mild toxicity due to its hemolytic activity (Vinay et al., 2014). Xiang et al. (2013) have shown that nanoparticles by themselves are not necessarily toxic if they are made of inert substances. Nevertheless, there are concerns regarding few nanoparticles showing varying degree of toxicity despite their vast application. For example, the inorganic carbon nanotubes are non-biodegradable and are reported to be toxic (Liu et al., 2009; Mutlu et al., 2010) and at the same time they are also reported to be biocompatible and non-toxic (Mitchell et al., 2007; Takagi et al., 2008). The use of carbon nanotubes for vaccine delivery in fish has also been attempted. Due to the discrepancies in toxicity reports there is a need to study and understand the proper mechanism of nanoparticles, which may provide a conclusive evidence on its applications in fish, animal, plants, and humans, which is limited at this stage. The toxicity of nanoparticles may vary in different species and also in combination with different antigens. Hence, the toxicity studies have to be conducted in species or taxonomically nearest species in which the nanoparticle has to be applied. The un-regulated application and the toxicity reports from very few in vitro studies may bias the perception on the use of nanoparticles and attract the unnecessary alarm in public and cast doubt on the science of nanomedicine (Yildirimir et al., 2011). With the increasing number of nanoparticle applications in recent times, the mechanisms will be clear and may change the perception either ways.

Cost effectiveness of nanovaccines

Nanovaccines are basically similar to general vaccines but they are much more advanced in terms of their delivery and efficacy due to the associated nanoparticles. They consist of the same inactivated pathogen or recombinant protein or a DNA construct but instead of being suspended in liquid it is contained in nanoparticles. The synthesis of nanoparticles costs very less than other adjuvant formulations. The most important thing is the requirement of cold chain for vaccine storage and transportation and world health organization (WHO) has estimated that the major cost of vaccine production goes into the cold chain (WHO, 2002). However, the nanoparticle-based vaccines do not require cold chain and it adds to the advantage and these thermostable nanovaccines may reduce the cost of production in a big way. Hence, the cost-effective and advanced nanovaccines have a great application in the aquaculture sector to prevent those infectious diseases against which the development of effective vaccines has been difficult.

Conclusion and future prospects

In the last decade there has been a remarkable advancement in nanotechnology and its application in biomedicine especially in vaccine delivery. Nanovaccines developed for aquacultured species has a fair share in this advancement. Nanoparticles have shown to enhance the immunogenicity of weak antigens and they provide many advantages over conventional adjuvant approaches like having better release kinetics, stability, and targeted delivery. This review summarizes the latest developments, current applications, toxicity issues, and cost effectiveness of nanoparticles in vaccine delivery to aquacultured species. Given the nature of aquaculture, the most preferred route of vaccination is oral delivery as it is not practical to inject every fish unlike other terrestrial species. Nanoparticles provide an opportunity to design vaccines that have gastrointestinal stability, a major requirement for oral vaccines. A recent study (Zhang et al., 2015) has demonstrated the designing of hybrid nanoparticles (intelligent shell), which provide extra protection in enzyme-rich stomach conditions and innovations like this will definitely solve the problems of mass vaccination in aquaculture in the coming days. Still there is a huge research gap and new forms of efficient nanoparticles like dendrimers, nanocapsules, mesoporous nanoparticles, and other forms are also available, which need to be explored for efficient vaccine delivery systems for aquacultured species. The fear still exists regarding the toxicity as the exact mechanism of action of nanoparticles still needs to be understood completely. The recent advancements and further studies on biocompatibility of these nanoparticles may change the perceptions and this will open new ways to counteract the deadly pathogens in aquaculture.

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