Fish and Shellfish Immunology 95 (2019) 213–219

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Enhanced efficacy of immersion vaccination in tilapia against columnaris disease by chitosan-coated “pathogen-like” mucoadhesive nanovaccines

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ARTICLE INFO

Keywords:
Immersion vaccination
Chitosan
Columnaris disease
Tilapia

ABSTRACT

Red tilapia (*Oreochromis* sp.) has become one of the most important fish in aquaculture. Bacterial infection caused by *Flavobacterium columnare*, the causative agent of columnaris disease, has been now identified as one of the most serious infectious diseases in farmed red tilapia and cause major financial damage to the producers. Among the effective prevention and control strategies, vaccination is one of the most effective approach. As the surface of living fish is covered by mucus and directly associated with the mucosal immunity, we therefore hypothesized that better adsorption on mucosal surfaces and more efficient vaccine efficacy could be enhanced biomimetic nanoparticles mimicking the mucoadhesive characteristic of live *F. columnare*. In this work, we describe an effective approach to targeted antigen delivery by coating the surface of nanoparticles with mucoadhesive chitosan biopolymer to provide “pathogen-like” properties that ensure nanoparticles binding on fish mucosal membrane. The physiochemical properties of nanovaccines were analyzed, and their mucoadhesive characteristics and immune response against pathogens were also evaluated. The prepared vaccines were nano-sized and spherical as confirmed by scanning electron microscope (SEM). The analysis of hydrodynamic diameter and zeta-potential also suggested the successful modification of nanovaccines by chitosan as indicated by positively charged and the overall increased diameter of chitosan-modified nanovaccines. *In vivo* mucoadhesive study demonstrated the excellent affinity of the chitosan-modified nanovaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. Following vaccination with the prepared nanovaccines by immersion 30 min, the challenge test was then carried out 30 and 60 days post-vaccination and resulted in high mortalities in the control. The relative percent survival (RPS) of vaccinated fish was greater than 60% for mucoadhesive nanovaccine. Our results also suggested that whole-cell vaccines failed to protect fish from columnaris infection, which is consistent with the mucoadhesive assays showing that whole-cell bacteria were unable to bind to mucosal surfaces. In conclusion, we could use this system to deliver antigen preparation to the mucosal membrane of tilapia and obtained a significant increase in survival compared to controls, suggesting that targeting mucoadhesive nanovaccines to the mucosal surface could be exploited as an effective method for immersion vaccination.

1. Introduction

According to 2015 UN Food and Agriculture Organization (GLOBEFISH - Analysis and information on world fish trade), Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* sp.) have increasingly recognized as one of the most important freshwater fish in aquaculture. Inevitably, several bacterial diseases can cause major financial damage to the producers of tilapia. Bacterial infection caused by *Flavobacterium columnare*, the causative agent of columnaris disease, has been now identified as one of the most serious infectious diseases in farmed tilapia [1]. *F. columnare* are gram negative, rod and slender filamentous bacterium with gliding motility and yellow rhizoid colony

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https://doi.org/10.1016/j.fsi.2019.09.064
Received 17 May 2019; Received in revised form 17 September 2019; Accepted 27 September 2019
Available online 01 October 2019
1050-4648/

formation [2] and colonizes the mucosal surfaces of fishes (gills and skin) in the initial steps of pathogenesis [3]. *F. columnare* infections may result in skin lesions, fin rot and gill necrosis, with a high degree of mortality, leading to severe economic losses [4]. The development of effective and affordable prevention and control strategies for columnaris infection is therefore warranted.

It is well established that vaccination is the most effective approach for prevention of infectious diseases in aquaculture. In fact, fish vaccines are mostly administered through major three routes of administration as bath or immersion, second through in-feed or oral and the third by injection. While immersion vaccination is more applicable, but this method suffers from low potency as the efficiency of uptake of antigens through the gills and skin are limited.

Nanoparticle platforms can be categorized as organic-based (e.g., lipid nanoparticles, biodegradable polymeric nanoparticles, and viral vectors), inorganic-based, or a hybrid combination of the two. The use of nanotechnology has been extensively exploited for controlled release and targeted delivery of drugs, vaccines, and biopharmaceuticals in order to improve their effectiveness for the prevention and treatment of human and animal diseases. It is well established that nanotechnology-based delivery system can play an important role in addressing the issue of inefficient targeting antigen to the action site which causes the administration of large doses of vaccine [5]. A number of previous studies investigating the different aspects related to nanoparticle vaccine and demonstrated their advantages over conventional vaccines. The use of nanotechnology-based delivery system has provided a tremendous opportunity to design new formulations of nanovaccine in order to effectively and selectively deliver antigens to appropriate sites, provide stability to antigens, and act as efficient adjuvants [6]. One purpose of this study was to exploit nanoencapsulation technology to enhance the efficacy of inactivated *F. columnare*.

Another purpose of this study was to exploit a mucoadhesive polymer-based delivery system to circumvent the issue of inefficient targeting antigen to fish mucosal surfaces. Chitosan (CS), sometimes known as deacetylated chitin found in the exoskeletons of crustaceans, is a natural polycationic linear polysaccharide [7]. Among polymers, chitosan is one of the most studied form of bioadhesive polymers [8] and has been extensively used in numerous applications in pharmaceutical and biomedical areas e.g. in drug delivery and tissue engineering due to its outstanding biological properties such as mucoadhesiveness, biocompatibility and biodegradability [9].

Taken together, we hypothesized that the efficacy of killed vaccines could be enhanced by nanoencapsulation in combination with incorporation of mucoadhesive characteristic. In this study, we prepared different formulations of nanovaccines as schematically shown in Fig. 1. The physiochemical properties of chitosan-complexed nanovaccine were analyzed, and their mucoadhesive characteristics and immune response against model antigen were also evaluated. Throughout this paper, the abbreviation WC, CS, NE and CS-NE will be used to refer to inactivated whole-cell *F. columnare* vaccines, polymeric (chitosan) nanovaccines, nanoemulsion vaccines and the hybrid nanoemulsion vaccines coated with mucoadhesive polymer chitosan, respectively.

2. Materials and methods

The procedures of animal experiments were approved by the Animal Ethics Committee of Chulalongkorn University, 1831020, and in accordance with animal ethics guidelines and approved protocols.

2.1. Fish and experimental conditions

Red tilapia (*Oreochromis* sp.) were used, with an average weight of 10 g. Fish were distributed into fiber tanks containing water under continuous aeration. Air and water temperatures were monitored daily and values were within acceptable ranges of 25–33 °C and 25–28 °C, respectively. Dissolved oxygen (DO) content and pH were examined weekly and were within ranges of 5.24–5.98 mg/L and 7.48–8.16, respectively.

2.2. Bacteria and nanovaccine preparation

Bacterial cultures used for nanovaccine preparation were grown in Tryptone Yeast Extract Salt (TYES) broth medium (pH 7.2) and incubated at 25–28 °C for 48 h [10]. In order to prepare inactivated vaccines, bacterial cells were collected by centrifugation at 3,000 g at 4 °C for 40 min, resuspended in phosphate-buffered saline (PBS) containing 0.2% formalin, and incubated at 4 °C for 20 h. Formalin-killed bacteria suspensions were washed three times by centrifugation and resuspended in PBS. Viable counts were determined by plating. After being adjusted to an identical number of bacterial cells based on an optical density-based approach (equivalent to 10^9 colony forming units (cfu)/mL predetermined by plating), an aliquot of bacterial cells was sonicated at 40% amplitude for 30 s and used to prepare different formulations of nanovaccine. Chitosan solution of 0.5% w/v concentration in 1% aqueous acetic acid was also prepared.

To prepare polymeric nanovaccines, an aliquot of sonicated bacterial cells (20% w/w) was mixed with 33% (w/w) of chitosan solution and 47% (w/w) of water. Stirring was continued for another 1 h at room temperature. To prepare nanoemulsion, an aliquot of sonicated bacterial cells (30% w/w) was mixed with 6% (w/w) of polyoxyethylene (20) sorbitan monolaurate, 2% (w/w) of medium chain triglycerides (Miglyol) and 62% (w/w) of water. The mixture was homogenized by sonicator probe at 40% amplitude for 5 min. To prepare chitosan-complexed nanoemulsion (hybrid), complexation of preformed nanoemulsions with chitosan was performed by adding 1% of small molecular weight (50–200 kDa; Sigma) chitosan (previously dissolved in a solution of 1% acetic acid) to the prepared nanoemulsion at a ratio of 1: 1 (v/v). The mixture was stirred for 1 h at room temperature.

2.3. Surface characterization of nanovaccines

Zeta potential of nanovaccine preparations were measured using a Malvern Instruments Zetasizer Nano ZX. The nanovaccine suspension was diluted 1,000 times in Deionized water before measurement. All measurements were performed at 25 °C. The data are given as mean ± SD based on the measurements of the samples from three replicates.

The morphology of nanovaccine preparations was observed using an environmental scanning electron microscope (E-SEM, S-3400, Horiba, Japan). The samples were diluted by distilled water at 1:50 ratio onto the carbon tape. The samples were then investigated at magnification of 5,000–20,000 times with electron beam energy of 20 kV.

2.4. Mucoadhesive characterization of nanovaccines

Fingerling tilapias (10 g) were divided into 4 groups; control, whole-cell vaccine, polymeric (CS) nanovaccine, nanoemulsion vaccines and hybrid CS-complexed nanoemulsion vaccine groups (5 fish each) with 3 replicates. Fish were immersed with 10^7 cfu/mL of vaccine preparations for 30 min. Following direct immersion and euthanasia, fish gills were harvested. Attachment of vaccines to mucosal surfaces as determined by the fluorescent signal of DAPI-stained vaccines was examined using a Nikon Eclipse TE2000-U fluorescence microscope. Fluorescence images were obtained by using 4X magnification and fluorescent setting. A second experiment was carried out to determine whether vaccines could be detected in the mucosal membranes. Following immersion, euthanasia and gill dissection, accumulation of DAPI-stained vaccines was observed using a bioluminescence imaging instrument (Bruker).

For quantitative measurement, 100 μl of Glo® lysis buffer (Promega) was added to 1 g of gill tissues, incubated for 10 min at 37 °C, and homogenized with PYREX® 3 mL Glass Pestle Tissue Grinder.

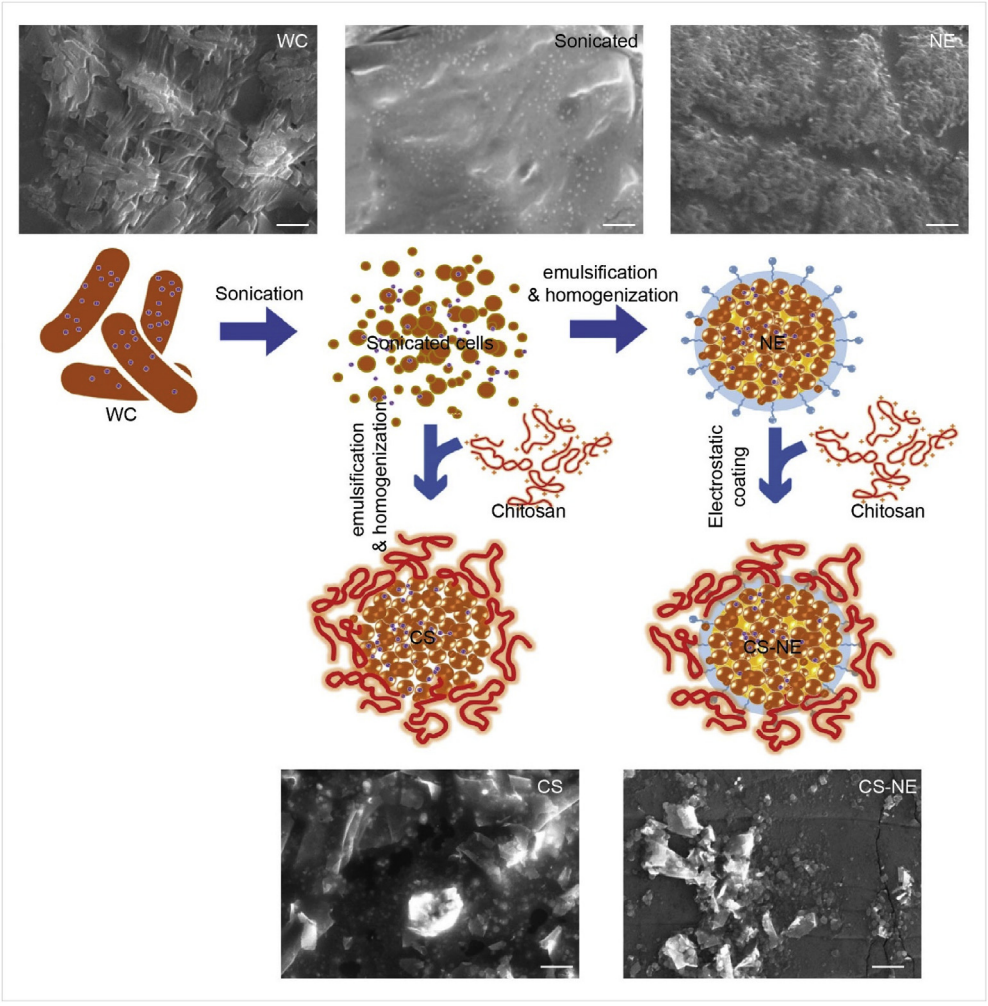

Fig. 1. Preparation and physical characteristics of different vaccine formulations used in this study. Schematic diagram of the prepared vaccines. Negatively charged vaccines prepared by formaldehyde inactivation were physically broken down, followed by reformation of nanoparticles with/without assembling with cationic chitosan polymers. SEM images of the surface morphology of different nanovaccine formulations in comparison with inactivated whole-cell vaccines are also shown. Scale bar = 10 μm.

Homogenized tissues were then centrifuged for 5 min at 10,000 g to remove cell debris. One hundred microliters of the supernatants was transferred to an opaque 96-well plate for fluorescence measurement. Fluorescence intensity was measured with a fluorescence plate reader at 358 nm/461 nm.

2.5. Vaccine efficacy

Fingerling tilapias (10 g) were divided into 5 groups; control, whole-cell vaccine, polymeric (CS) nanovaccine, nanoemulsion vaccines and hybrid CS-complexed nanoemulsion vaccine groups (25 fish each) with 3 replicates. Fish were immersed in aerated 2 L bath solutions containing 20 mL (1:100 dilution) for 30 min. Control immersion baths was prepared by 2 L sterile water. After vaccination, fish were transferred to fiber tanks containing water under continuous aeration. At 30 and 60 days after immersion vaccination, Fish were challenged with 1×10^6 CFU/mL lethal concentration of a virulent strain of *F. columnare* for 1 h. Cumulative mortality and survival rate were recorded for 10 days after immersion challenge.

2.6. Statistical analysis

GraphPad Prism software (version 5.0) was used to generate graphs and perform statistical analyses. One-way analysis of variance, or repeated measures analysis of variance, followed by Tukey post-hoc tests were used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant and denoted as follows: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. Survival curves were generated for the vaccinated fish and unvaccinated fish. The numbers of fish which died after challenge test were recorded. Relative percent survival (RPS) was calculated as 1 - (mortality rate of vaccinated fish/mortality rate of control fish) x100.

3. Results

3.1. Physicochemical characteristics of different nanovaccine formulations

We analyzed the zeta potential, size, and the appearance of the prepared nanovaccines. As shown in Table 1, we observed the zeta potential shifts from a negative value for whole-cell vaccines and uncomplexed NE, to a positive value for the CS vaccines and NE following complexation with CS polymers (CS-NE). These data proved the positive charge of the cationic nanovaccines in contrast to the negatively charged surface of the uncomplex vaccines. Formation of nanovaccines was also confirmed by the measurement of particle size. Size measurement of different formulations of nanovaccines in Table 1 revealed that CS nanovaccine has an average diameter of 350 ± 50 nm. Our results also showed that CS-NE has an average diameter of 2-fold

Table 1
Physicochemical properties of nanovaccines after formulations.

Formulation	Average diameter (nm)	Zeta potential (mV)	PDI
WC	1,900 ± 400	−15 ± 5.0	0.42
CS	350 ± 50	27.5 ± 2.5	0.12
NE	175 ± 25	−22.5 ± 2.5	0.14
CS-NE	350 ± 50	28.5 ± 6.5	0.19

Values were the means of three replicate samples. The data were presented as mean ± SD.

greater than the uncomplexed NE.

3.2. Mucoadhesiveness of the prepared nanovaccines

The affinity of different vaccine preparations toward mucosal surfaces of fish gills was studied using DAPI-stained *F. columnare*. Quantification of fluorescent signal in gill tissues after tissue lysis showed that a significant higher mean fluorescence intensity (MFI) were achieved with CS nanovaccines and CS-NE vaccines compared to whole-cell vaccine and nanoemulsion vaccines. As shown in Fig. 2a, treatment with CS nanovaccines and CS-NE vaccines resulted in 4 to 5-fold increase of fluorescent signal compared to control (non-treated) group, respectively. Fluorescence microscopy revealed that a large number of CS nanovaccines and CS-NE nanovaccines can bind to fish gills, whereas a few particles were observed on gills of fish immerged in water containing whole-cell vaccines or NE vaccines (Fig. 2b). Consistently, bioluminescence imaging revealed that incorporation of nanovaccines with chitosan biopolymer mediated efficient attachment to mucosal surfaces, as indicted by higher fluorescent intensity than of naked vaccines (Fig. 2c).

3.3. Protective effect of mucoadhesive vaccines against flavobacterium infection

Vaccinated and control fish were held for 30 and 60 days following bath vaccination before they were challenged with virulent *F. columnare*. Time course for bath immunization and challenge is shown in Fig. 3a. There was no fish died after the vaccination. A positive effect of vaccination is determined by a relative percent survival (RPS) greater than 60%. At 30 days post vaccination with all vaccine formulations, the RPS were greater than 60%, as shown in Table 2. In this study, mortality in an equivalent group of non-vaccinated fish was 87%. The prolonged protective effect could be also observed in fish vaccinated with nanovaccines at 60 days post vaccination (Table 2). However, a loss of protection after initial effectiveness was observed in a group vaccinated with whole-cell bacteria as shown by mortality in this group was lower than 50%. Percentage survival after bath challenge of vaccinated and control groups is shown in Fig. 3b. We observed at least one of the following clinical signs in all the moribund and dead fish; hemorrhage, lesion on the trunk, and/or eroded tail (Fig. 3c). Moreover, chitosan polymer alone fails to protect tilapia from columnaris infection. Following immersion with the chitosan polymer by immersion 30 min, the challenge test was then carried out 30 and 60 days post-immersion and resulted in high mortalities (Supplementary Table 1).

4. Discussion

Among the effective prevention and control strategies, vaccination is one of the most effective approach. Vaccines for use in fish against infectious pathogens can be broadly categorized as traditional and modern vaccines. The former includes killed vaccines and attenuated vaccines while the latter includes recombinant technology vaccines and synthetic peptide vaccines as well as DNA vaccines, all of which are in progress around the world [11,12]. In general, all of these strategies have shown advantages and disadvantages. Despite several efforts to develop effective vaccines against a large number of diseases using

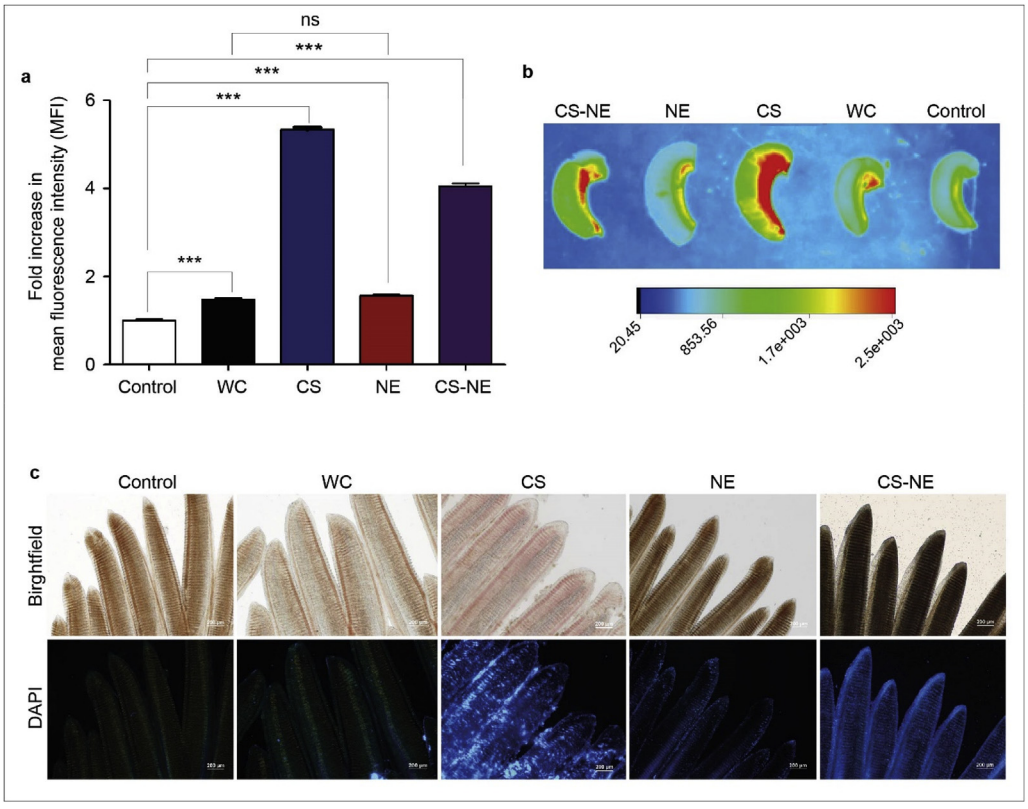

Fig. 2. *In vivo* accumulation of mucoadhesive vaccines in gill tissues after direct immersion. a) Quantitative analysis of DAPI-stained vaccines in fish gill tissues. Experiments were performed in triplicate and data presented as fold-increase in mean fluorescence intensity (MFI) compared with the control (non-vaccinated fish). **b**) Fluorescence imaging of fish gills following direct immersion of different formulations of nanovaccines compared to whole-cell vaccines. (**c**) Representative microscopic fluorescence images of nanovaccines in fish gill slices after direct immersion as examined by fluorescence microscopy. Scale bar = 200 μm.

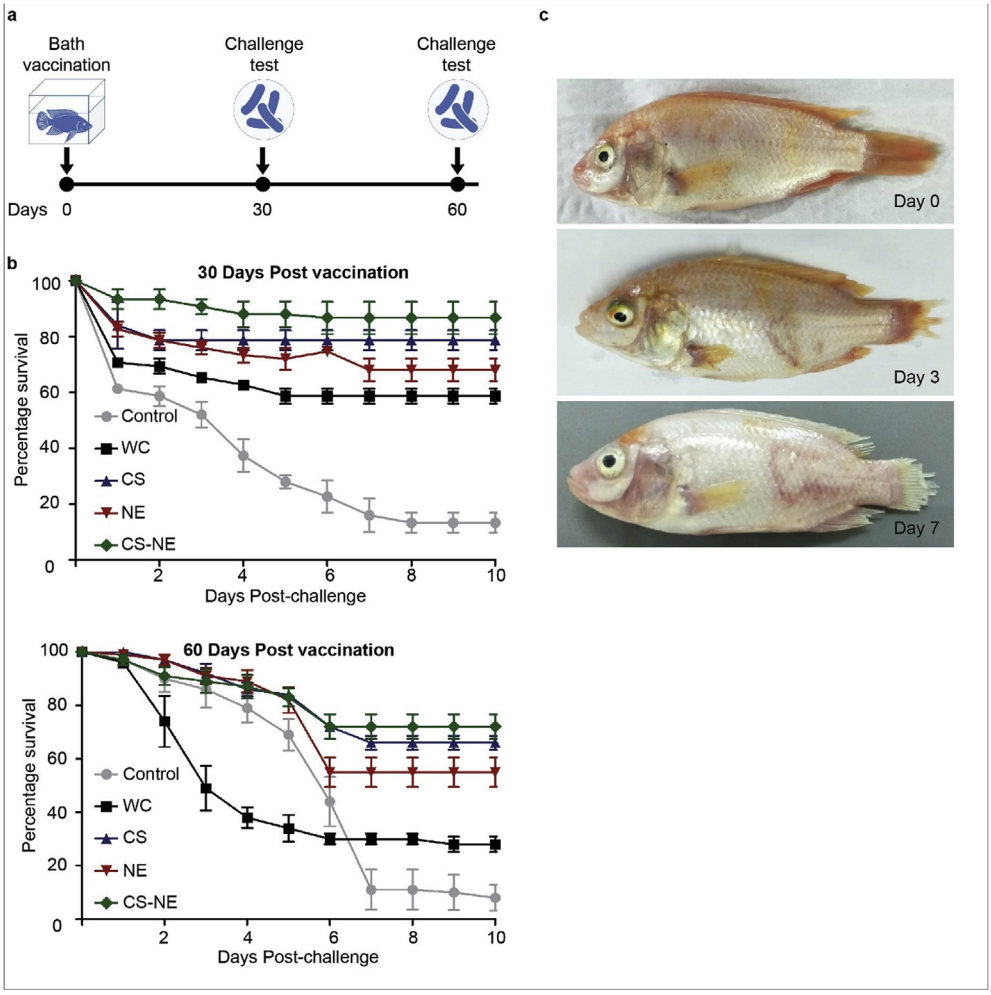

Fig. 3. Vaccine efficacy. (a) Time course for immersion immunization and challenge test. (b) Percentage survival after bath challenge of vaccinated and control groups. The survival rates following challenge with 1×10^6 CFU/mL *F. columnare* are presented. (c) Clinical signs of columnaris disease following bath challenge with *F. columnare*.

recombinant vaccines and recombinant DNA technology, the inherent limitation of these modern antigens is their low immunogenicity in comparison to the more traditional vaccines. The poor immunogenicity frequently observed in recombinant antigens is associated with a lack of exogenous immune activating components [13].

At the present, most of licensed aquaculture vaccines are in the form of live attenuated, killed/inactivated microorganisms [14,15]. Traditional vaccines are generally more effective than modern vaccines. The explanation behind this fact is that protection is mediated by the combination of multiple antigens composed of lipopolysaccharides, lipoproteins, complex polysaccharides as well as proteins [16–18]. Traditional vaccines that express these multiple antigens thus provide the most efficacious immunity superior to recombinant vaccines. As compared to inactivated vaccines, live attenuated vaccines can be highly effective [19,20]. However, their potential risk of reversion of the microorganism for a more virulent phenotype can occur [15,21]. This major concern has become a limitation for their use in aquaculture including in Thailand. Although killed/inactivated vaccines may be less effective than attenuated vaccines, they are typically safer.

Nanotechnology-based delivery system has been extensively used in vaccine development as it is effortless to deliver, protect the antigen from degeneration and is found to be efficient with a single dose resulted from slow release of the encapsulated antigen [22]. As mentioned earlier, we hypothesized that the efficacy of killed vaccines could be enhanced by nanoencapsulation technology. We evaluated the average size and zeta potential to characterize the nanovaccine prepared from formalin-killed and sonicated *F. columnare* via the emulsification and homogenization technique. Our results also showed that the prepared nano-sized vaccines are well-dispersed in water and provided excellent protective effect against columnaris disease following immersion vaccination as compared to inactivated whole-cell bacteria. This result could be explained by the finding that smaller nanoparticles ranging from 1 to 100 nm can be easily delivered to lymph nodes because they can be readily internalized by dendritic cells and retained for a longer period of time at the vaccine administration site [23]. Moreover, the use of nano-sized vaccines improves immunogenicity in the absence of adjuvants such as alum, which are inflammatory mediators.

Gills, skin, and gut are important organs that directly associated with the mucosal immunity of teleost fish and play a very important part of the fish immune defenses, protecting the body from the first encounter of infectious pathogens [24]. The external constituent of skin, gills, and gut is a mucous gel which forms a layer of a gel-like substance covering the epithelial cells [25]. The fish mucus is mainly composed of water and glycoproteins, containing a vast majority of mucins, high molecular weight negatively charged oligosaccharides [26,27].

As colonization of the mucosal surfaces of fish skin and gills is the first step of *F. columnare* infection [3], we therefore hypothesized that better adsorption on mucosal surfaces and more efficient vaccine efficacy could be enhanced by biomimetic nanoparticles mimicking the

Table 2
Average percent mortality of tilapias after bath challenge (30 or 60 days post vaccination) with *F. columnare*.

Group	Replicate tank	*n*	Average % mortality	Average % survival	RPS
Control (30 days)	1	25	87	13	–
	2	25			
	3	25			
WC vaccine (30 days)	1	25	41	59	53
	2	25			
	3	25			
CS vaccine (30 days)	1	25	21	79	76
	2	25			
	3	25			
NE vaccine (30 days)	1	25	32	68	63
	2	25			
	3	25			
CS-NE vaccine (30 days)	1	25	13	87	85
	2	25			
	3	25			
Control (60 days)	1	25	92	8	–
	2	25			
	3	25			
WC vaccine (60 days)	1	25	72	28	22
	2	25			
	3	25			
CS vaccine (60 days)	1	25	34	66	63
	2	25			
	3	25			
NE vaccine (60 days)	1	25	45	55	51
	2	25			
	3	25			
CS-NE vaccine (60 days)	1	25	28	72	70
	2	25			
	3	25			

Immersion vaccination with 1×10^6 CFU/mL *F. columnare* for 30 min immersion exposure.

mucoadhesive characteristic of live *F. columnare*. Our results also confirmed that the positively charged nanovaccines increased attachment to fish gill tissues mainly by an efficient binding of nanovaccines to the negatively charged mucosal membranes. Another possible explanation for enhanced protective effect could be the adjuvant ability of chitosan [28–30]. Chitosan has been widely studied for its immunogenic activities, especially via the mucosal routes [31–33].

Despite these promising results, a basis for the safety of novel vaccines must be established before regulatory agencies approve initiation of animal clinical trials. Further research should be undertaken in farmed tilapia in order to identify both intrinsic toxicity of the product and immunotoxicity arising from the host immune response to the new vaccine. Moreover, some related clinical parameters are required to be measured and monitored over a period of time, such as Average Daily Gain (ADG), Feed Conversion Ratio (FCR).

5. Conclusion

The strategy, as presented here, is an improved version of inactivated nanovaccine surfaced modified with chitosan biopolymers and targeted to the mucosal membrane of tilapia. Specifically, we reported here the preparation of mucoadhesive nanovaccines via the emulsification and homogenization method as well as their physicochemical and biological properties. The analysis of SEM image and zeta-potential also suggested the successful modification of nanovaccines by chitosan. *In vivo* mucoadhesive studies demonstrated the excellent affinity of the chitosan-complexed nanovaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. By taking advantage of the unique characteristics of the fish mucus, the present study demonstrated that targeting mucoadhesive vaccines to the fish gill mucosal surface could be exploited as an effective method for immersion vaccination. Interestingly, our data confirmed that the complexation of nanovaccines with cationic chitosan polymers generates positively charged vaccine complexes. As a result, biomimetic nanoparticles mimicking the mucoadhesive characteristic of live *F. columnare* can help achieve better adsorption on mucosal surfaces and more efficient vaccine efficacy. Taken together, our study demonstrated the feasibility of mucoadhesive nanoparticle as an effective delivery method for an inactivated vaccine against infectious *F. columnare* in Tilapia by immersion vaccination.

Author contributions

N.P., T.Y., and C.R. were involved in the design and supervision of all experiments. S.K (Sirikorn), K.S., J.K. and S.S. were involved in conducting physicochemical experiments. N.P., T.Y., and C.R. were involved in the design and supervision of animal experiments. S.K. (Sirikorn), K.S., S.K. (Somrudee), N.N., and K.N. were involved in conducting the biological experiments including *in vivo* studies. S.K. (Sirikorn), N.P., T.Y., and C.R. performed the statistical analyses and wrote the manuscript text. All authors reviewed the manuscript.

Declaration of competing interest

The authors report no conflicts of interest in this work.

Acknowledgments

This work was supported by the grant from the 90th Anniversary of Chulalongkorn University Scholarship; GCUGR1125622098D, Ratchadaphiseksomphot Endowment Fund; CU-GR_61_013_31_003, National Nanotechnology Center; P1752586 and P1751449, and Thailand Graduate Institute of Science and Technology Scholarship; SCA-CO-2560-4578-TH, National Science and Technology Development Agency (NSTDA, Thailand). Faculty of Veterinary Science, Chulalongkorn University, is also acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.064.

References

[1] P. Wonmongkol, et al., Genetic parameters for resistance against Flavobacterium columnare in nile tilapia Oreochromis niloticus (linnaeus, 1758), J. Fish Dis. 41 (2018) 321–328.
[2] H. Dong, B. LaFrentz, N. Pirarat, C. Rodkhum, Phenotypic characterization and genetic diversity of Flavobacterium columnare isolated from red tilapia, Oreochromis sp., in Thailand, J. Fish Dis. 38 (2015) 901–913.
[3] C.A. Shoemaker, B.R. LaFrentz, Growth and survival of the fish pathogenic bacterium, Flavobacterium columnare, in tilapia mucus and porcine gastric mucin, FEMS Microbiol. Lett. 362 (2015), https://doi.org/10.1093/femsle/fnu060.
[4] T.P. Loch, M. Faisal, Emerging flavobacterial infections in fish: a review, J. Adv. Res. 6 (2015) 283–300.
[5] M. Aklakur, M. Asharf Rather, N. Kumar, Nanodelivery: an Emerging avenue for nutraceuticals and drug delivery, Crit. Rev. Food Sci. Nutr. 56 (2016) 2352–2361.
[6] T.N. Vinay, et al., Recent advances in application of nanoparticles in fish vaccine delivery, Rev. Fish. Sci.Aquacult. 26 (2018) 29–41.
[7] T.M. M Ways, W.M. Lau, V.V. Khutoryanskiy, Chitosan and its derivatives for application in mucoadhesive drug delivery systems, Polymers 10 (2018) 267.
[8] C. Saikia, P. Gogoi, T. Maji, Chitosan: a promising biopolymer in drug delivery applications, J. Mol. Genet. Med. S 4 (2015) 006.
[9] R.C.F. Cheung, T.B. Ng, J.H. Wong, W.Y. Chan, Chitosan: an update on potential biomedical and pharmaceutical applications, Mar. Drugs 13 (2015) 5156–5186.
[10] L. Grabowski, S. LaPatra, K. Cain, Systemic and mucosal antibody response in tilapia, Oreochromis niloticus (L.), following immunization with Flavobacterium columnare, J. Fish Dis. 27 (2004) 573–581.
[11] B.E. Correia, et al., Proof of principle for epitope-focused vaccine design, Nature 507 (2014) 201.
[12] O.A. Dellagostin, et al., Recombinant vaccines against leptospirosis, Hum. Vaccine 7 (2011) 1215–1224.
[13] S. Jorge, O.A. Dellagostin, The development of veterinary vaccines: a review of

traditional methods and modern biotechnology approaches, Biotechnol. Res. Innovation 1 (2017) 6–13.
[14] S. McVey, J. Shi, Vaccines in veterinary medicine: a brief review of history and technology, Vet. Clin. Small Anim. Pract. 40 (2010) 381–392.
[15] M. Unnikrishnan, R. Rappuoli, D. Serruto, Recombinant bacterial vaccines, Curr. Opin. Immunol. 24 (2012) 337–342.
[16] B.R. LaFrentz, S.E. LaPatra, D.R. Call, K.D. Cain, Isolation of rifampicin resistant Flavobacterium psychrophilum strains and their potential as live attenuated vaccine candidates, Vaccine 26 (2008) 5582–5589.
[17] C.A. Shoemaker, P.H. Klesius, J.D. Drennan, J.J. Evans, Efficacy of a modified live Flavobacterium columnare vaccine in fish, Fish Shellfish Immunol. 30 (2011) 304–308.
[18] C.A. Shoemaker, P.H. Klesius, J.J. Evans, C.R. Arias, Use of modified live vaccines in aquaculture, J. World Aquac. Soc. 40 (2009) 573–585.
[19] C. da Costa, B. Walker, A. Bonavia, Tuberculosis vaccines–state of the art, and novel approaches to vaccine development, Int. J. Infect. Dis. 32 (2015) 5–12.
[20] C. Rizzi, et al., Vaccination with a BCG strain overexpressing Ag85B protects cattle against Mycobacterium bovis challenge, PLoS One 7 (2012) e51396.
[21] Y. Shimoji, et al., Erysipelothrix rhusiopathiae YS-1 as a live vaccine vehicle for heterologous protein expression and intranasal immunization of pigs, Infect. Immun. 70 (2002) 226–232.
[22] A.A. Walters, C. Krastev, A.V. Hill, A. Milicic, Next generation vaccines: single-dose encapsulated vaccines for improved global immunisation coverage and efficacy, J. Pharm. Pharmacol. 67 (2015) 400–408.
[23] S.G. Reed, M.T. Orr, C.B. Fox, Key roles of adjuvants in modern vaccines, Nat. Med. 19 (2013) 1597.
[24] F.A. Guardiola, A. Cuesta, E. Abellán, J. Meseguer, M.A. Esteban, Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish, Fish Shellfish Immunol. 40 (2014) 24–31.
[25] S. Koshio, Immunotherapies targeting fish mucosal immunity–Current knowledge and future perspectives, Front. Immunol. 6 (2016) 643.
[26] M. Grosell, A.P. Farrell, C.J. Brauner, Fish Physiology: the Multifunctional Gut of Fish 30 Academic Press, 2010.
[27] J. Perez-Vilar, Mucin granule intraluminal organization, Am. J. Respir. Cell Mol. Biol. 36 (2007) 183–190.
[28] E.C. Carroll, et al., The vaccine adjuvant chitosan promotes cellular immunity via DNA sensor cGAS-STING-dependent induction of type I interferons, Immunity 44 (2016) 597–608.
[29] H. Chang, et al., Comparison of adjuvant efficacy of chitosan and aluminum hydroxide for intraperitoneally administered inactivated influenza H5N1 vaccine, DNA Cell Biol. 29 (2010) 563–568.
[30] W.R. Chen, Chitin, chitosan, and glycated chitosan regulate immune responses: the novel adjuvants for cancer vaccine, Clin. Dev. Immunol. 2013 (2013).
[31] B.C. Baudner, et al., The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines, Vaccine 21 (2003) 3837–3844.
[32] Y. Xia, et al., Chitosan-based mucosal adjuvants: sunrise on the ocean, Vaccine 33 (2015) 5997–6010.
[33] L. Zeng, Mucosal adjuvants: opportunities and challenges, Hum. Vaccines Immunother. 12 (2016) 2456–2458.