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The potential of mucoadhesive polymer in enhancing efficacy of direct immersion vaccination against *Flavobacterium columnare* infection in tilapia

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ABSTRACT

Vaccination is the most effective approach for prevention of infectious diseases in aquaculture. Although immersion vaccination is more applicable compared to in-feed/oral administration and injection, this method suffers from low potency as the efficiency of uptake of antigens through mucosal membranes is limited. In this study, we have successfully developed a mucoadhesive vaccine delivery system to enhance the efficacy of direct immersion vaccination against *Flavobacterium columnare*, the causative agent of columnaris disease in red tilapia. A formalin-killed negatively charged, bacterial cell suspension was used to prepare a mucoadhesive vaccine by electrostatic coating with positively charged chitosan. Our results demonstrate that the chitosan-complexed vaccine greatly increases its mucoadhesiveness, thus increasing the chances of vaccine uptake by the gill mucosa and improving the protection obtained against columnaris infection. The surface charge of the chitosan-complexed vaccine was altered from anionic to cationic after chitosan modification. Tilapia were vaccinated with the prepared chitosan-complexed vaccine by immersion. The challenge test was then carried out 30 and 60 days post vaccination, which resulted in a high level of mortalities in the non-vaccinated and uncomplexed vaccine groups. A high relative percentage survival (RPS) of vaccinated fish was noted with the mucoadhesive vaccine. Our results indicated that the naked vaccine failed to protect the fish from columnaris infection, which is consistent with the mucoadhesive assays performed during the study showing that the naked vaccine was unable to bind to mucosal surfaces. This system is therefore an effective method for immersion vaccination in order to deliver the antigen preparation to the mucosal surface membrane of the fish.

1. Introduction

Tilapia (*Oreochromis* sp.) is one of the most important fish species produced in fish farming [1]. 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 [2]. *F. columnare* is a Gram-negative, rod and slender filamentous bacterium with gliding motility and yellow rhizoid colony formation [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].

It is well established that vaccination is the most effective approach for prevention of infectious diseases in aquaculture [5]. Aquaculture vaccines are roughlyadministered through major three routes, i.e. bath or immersion, in-feed or oral, and injection [6]. While immersion vaccination is the most applicable mode of delivery of these routes of administration, this method suffers from low potency as the efficiency of antigen uptakes through the gills and skin is limited [7].

Chitosan (CS), sometimes known as deacetylated chitin found in the exoskeletons of crustaceans, is a natural polycationic linear polysaccharide that exhibits mucoadhesive properties [8]. Among polymers, chitosan has been exploited for the design of mucoadhesive dosage

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forms due to its excellent biocompatibility and biodegradability [9].

The external constituent of skin, gills, and gut is a mucous gel secreted by various epidermal or epithelial mucus cells which forms a layer of a gel-like substance covering the epithelial cells [10]. More importantly, these organs are directly associated with the mucosal immunity of fish [11]. The fish mucus is mainly composed of water and glycoproteins, containing vast amount of mucins, high molecular weight negatively charged oligosaccharides [12,13]. Since fish gills are considered a mucosal surface associated with the mucosal immunity, targeting mucoadhesive vaccines to the mucosal surface could be exploited as an effective method for immersion vaccination. It has been suggested that electrostatic force attraction is crucial for the mucoadhesive mechanism, which is affected by the complexation between positively charged polymer and negatively charged materials such as cell surface and mucin in a biomembrane environment [14]. Therefore, we hypothesized that a mucoadhesive polymer could be exploited to deliver antigen preparation to mucosal membranes of tilapia.

The main overall aim of this study was to investigate the application of chitosan to facilitate efficient delivery of inactivated vaccines to fish mucosal surfaces. In this study, we prepared chitosan-complexed vaccines as schematically shown in Fig. 1. The physiochemical property of chitosan-complexed vaccines was analyzed, and their mucoadhesive characteristics and protective effect against columnaris disease were evaluated. Throughout this paper, the abbreviation CS-vaccine will be used to refer to inactivated *F. columnare* vaccines complexed with mucoadhesive polymer chitosan.

2. Materials and methods

Ethics Statement Care of laboratory animals and animal experimentation were conducted in accordance with animal ethics guidelines and approved protocols. All animal experiments were approved by the Animal Ethics Committee of Chulalongkorn University, 1831020. Upon termination of the study, all fish were euthanized according to appropriate guidelines.

2.1. Fish and experimental conditions

Healthy red tilapia (*Oreochromis* sp.) with an average weight of 10 g were purchased from a tilapia breeding farm, Thailand and used for the experiment. Fish were distributed into 800 L fiber tanks containing water under continuous aeration and with continuous water flow (80% water change per day). Air and water temperatures were measured daily and were 25–33 °C and 25–28 °C, respectively. Dissolved oxygen (DO) content and pH were measured weekly using a DO meter and pH meter, and values were within acceptable ranges of 5.24–5.98 mg $L^{-1}$and 7.48–8.16.

2.2. Bacteria and vaccine preparation

Bacterial cultures (*F. columnare* isolated from red tilapia, *Oreochromis* sp., in Thailand) [15] used for vaccine preparation and challenge test were grown in Tryptone Yeast Extract Salt (TYES) broth medium (pH 7.2) and incubated at 25–28 °C for 48 h [16]. In order to prepare inactivated vaccines, bacterial cells were harvested 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 re-suspended in PBS (adjusted to 10^9 colony forming units (cfu/mL)). Subsequently, an aliquot of bacterial cells was used to complex with chitosan (Sigma-Aldrich) by adding 1% w/v of chitosan (previously dissolved in a solution of 1% acetic acid) to the prepared formalin-killed vaccine at a ratio of 1: 1 (v/v). The mixture was stirred for 30 min at 25 °C.

2.3. Surface characterization of the chitosan-complexed vaccines

Zeta potential of vaccine preparations were determined using a Zetasizer Nano ZX (Malvern Instruments). The vaccine solution was diluted 1000 times in DI water before measurement. All measurements were performed at 25 °C. The data are given as mean ± SEM based on the measurements of three replicate samples.

The morphology of vaccine preparations was observed using an environmental scanning electron microscope (E-SEM, S-3400, Horiba). The samples were diluted by distilled water at ratio of 1:50 onto the surface of carbon tape. The samples were then observed at of 5000–20,000× magnifications with electron beam energy of about 20 kV.

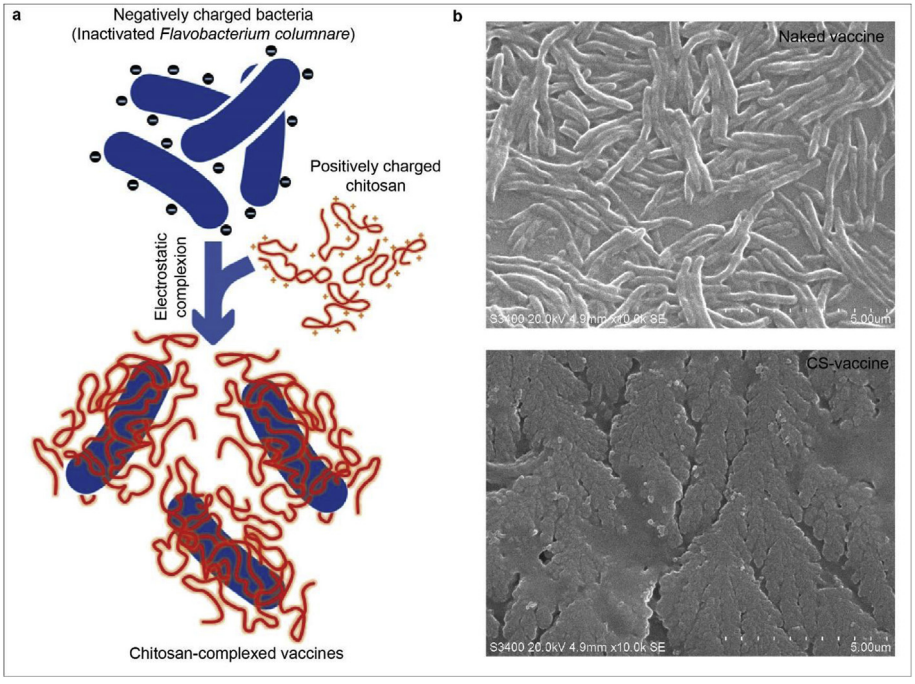

Fig. 1. Surface characteristics of the chitosan-complexed vaccines. a). Schematic diagram of the chitosan-complexed vaccines. Negatively charged vaccines prepared by formaldehyde inactivation were electrostatically assembled with cationic chitosan polymers to form chitosan-vaccine complexes. **b)**. SEM images of the surface morphology of the chitosan-complexed vaccines in comparison with naked vaccines.

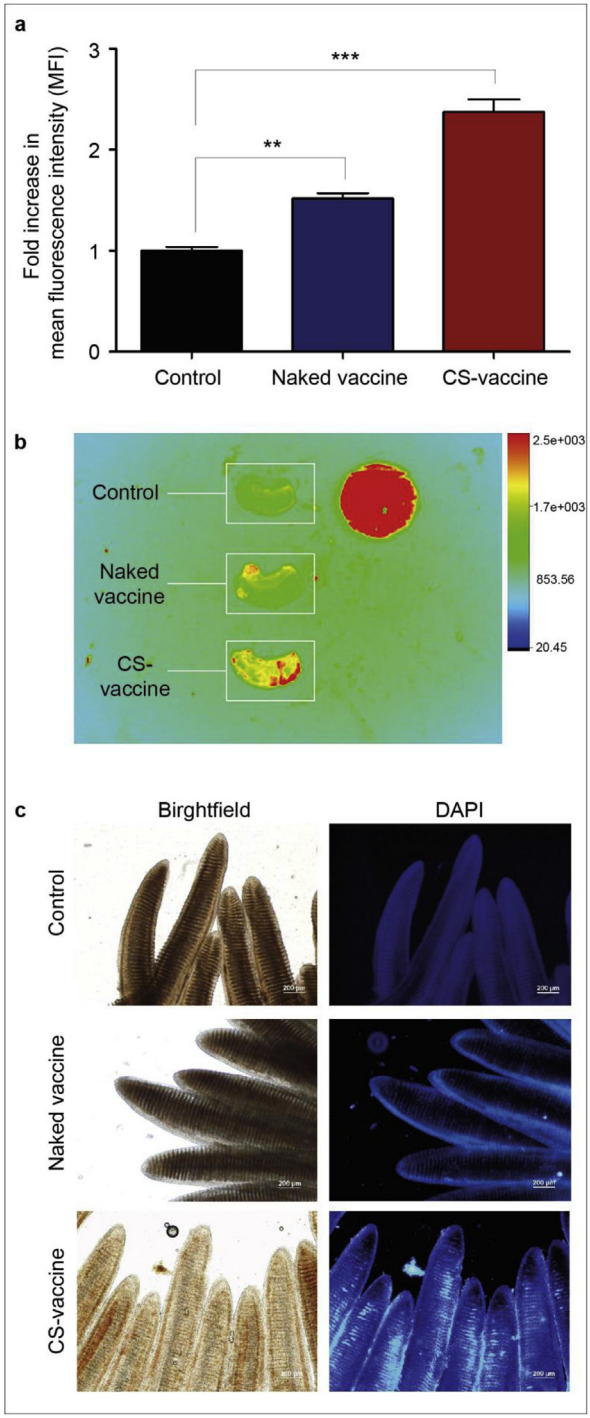

Fig. 2. Accumulation of mucoadhesive vaccines in gill tissues after direct immersion vaccination. a) Quantitative analysis of DAPI-stained vaccines in fish gill tissues. Experiments were performed in triplicate and data presented as fold-increase in mean fluorescence intensity (MFI) compared with the control (non-vaccinated fishes). **b**) Bioluminescence imaging of fish gills following direct immersion of naked vaccines and CS-vaccines. **c**) Representative microscopic fluorescence images of vaccines complexed with chitosan in fish gill after direct immersion as examined by fluorescence microscopy. Scale bar = 200 μm.

2.4. Mucoadhesive properties of the chitosan-complexed vaccines

DAPI (4′,6-diamidino-2-phenylindole) (Sigma-aldrich) solution was added to the formalin-killed bacteria suspension (final concentration: 0.5 μg/mL) and incubated in the dark at room temperature for 5 min. The suspension was washed three times by centrifugation and re-suspended in PBS and the stained cells were used to prepare the chitosan-complexed vaccines. Fingerling tilapias (10 g) were divided into 3 groups; control, naked vaccine, and CS-vaccine groups (5 fishes each) in 3 replicate tanks. Fish were immersed in 10^7 cfu/mL of vaccine preparations for 30 min. Following direct immersion and euthanasia by rapid chilling (2°–4 °C) until loss of orientation and operculum movements and subsequent holding times in ice-chilled water, fish gills were harvested. Attachment of vaccines to mucosal surfaces was examined by determining the fluorescent signal of DAPI-stained vaccines using a Nikon Eclipse TE2000-U fluorescence microscope. Photographic images were obtained by using 4× magnification and fluorescent setting with an excitation max of 358 nm and emission max of 461 nm. Fish gills were also observed using a bioluminescence imaging instrument (Bruker). To quantity the attached bacteria, 100 μl of Glo® lysis buffer (Promega) was added to 1 g of gill tissue, incubated for 10 min at 37 °C, and homogenized with PYREX® 3 mL Glass Pestle Tissue Grinder. Homoginized tissues were then centrifuged for 5 min at 10,000 g to remove debris. One hundred microliters of the supernatants was transferred to an opaque 96-well plate for fluorescence measurement. Fluorescence was read with a fluorescence plate reader at 358 nm/461 nm.

2.5. Vaccine efficacy

Tilapia Fingerlings (10 g) were divided into 3 groups; control, naked vaccine, and CS-vaccine groups (15 fish each) in 2 replicates. Fish were immersed with 10^7 cfu/mL of vaccine preparations for 30 min. At 30 and 60 days after immersion vaccination, fishes were challenged with 1×10^6 CFU/mL concentration of a virulent strain of *F. columnare* for 1 h (the same isolate used to prepared the vaccine as previously described [15]. Cumulative mortality and survival rate were recorded for 10 days after immersion challenge. Columnaris disease caused by F. columnare was confirmed by clinical signs of necrotic gills, fin rot, skin erosion or necrotic muscle, followed by a characteristic rhizoid pattern of growth on a low nutrient agar medium [17].

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 percentage survival (RPS) was calculated as 1 - (mortality rate of vaccinated fish/mortality rate of control fish) [18].

3. Results

3.1. Surface characteristics of the chitosan-complexed vaccines

Fig. 1b shows the SEM image of naked vaccines and chitosan-complexed vaccines. The morphology of the chitosan-complexed vaccines exhibited well-formed vaccines complexed with chitosan forming a rough surface. When we analyzed the zeta potential of the CS-complexed vaccines, we observed that the zeta potential shifted from a negative value of -6.44 ± 1.00 mV for naked vaccines, to a positive value of $+11.62 \pm 2.29$ mV for the CS-complexed vaccines. These data proved the positive charge of the CS-complexed vaccines in contrast to the negatively charged surface of the naked vaccines.

3.2. Mucoadhesive property of the chitosan-complexed vaccines

The affinity of chitosan-complexed vaccines toward a mucosal

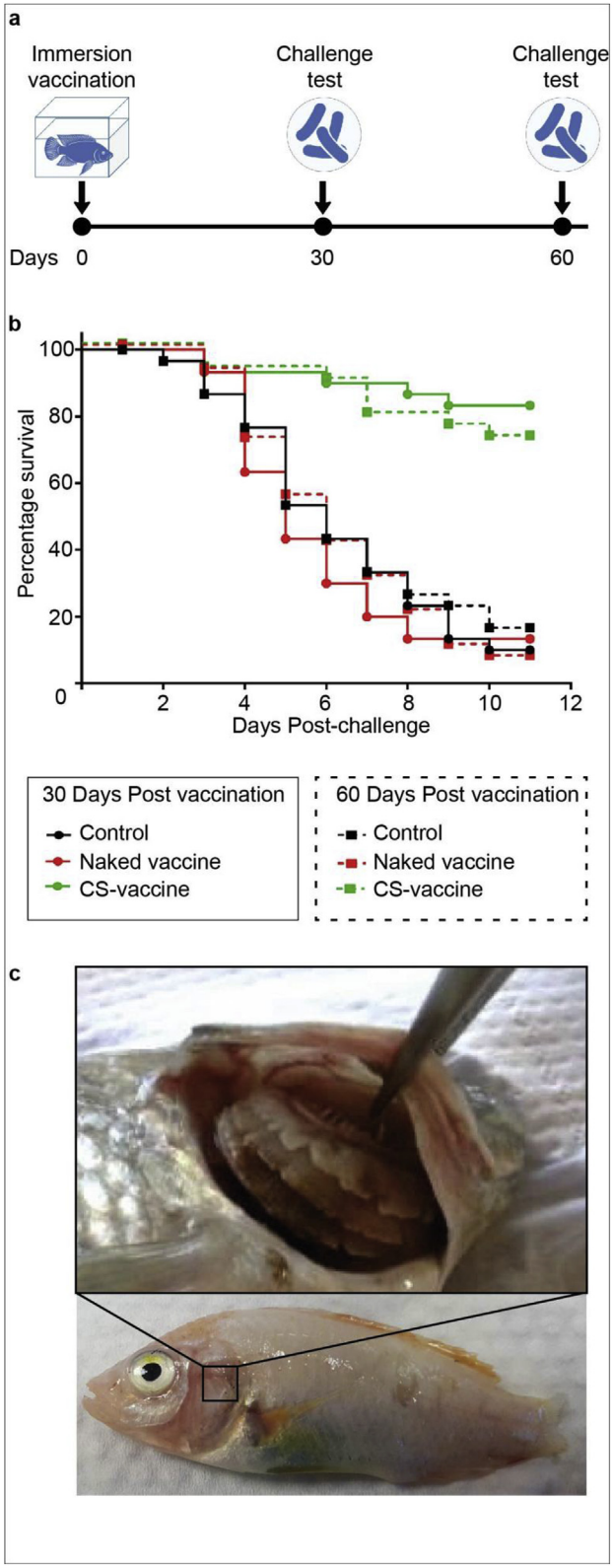

Fig. 3. Vaccine efficacy. (**a**) Time course for bath immunization and challenge. (**b**) Percentage survival after bath challenge of vaccinated and control groups. The survival rates following challenge with 1×10^6 CFU/mL *F. columnare* are presented. **(c)** Clinical signs of columnaris infection following bath challenge with *F. columnare*. Diseased Tilapia showing prominent yellowish deposits in the gills.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface of fish gill 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-vaccines compared to naked vaccines. As shown in Fig. 2a, treatment with CS-vaccines and naked vaccines resulted in ~2- and ~1.25-fold increase of fluorescent signal compared to the control (non-treated) group, respectively. Fluorescence microscopy revealed a large number of CS-vaccines attached to fish gills. In contrast, a few particles of naked vaccines were observed on gills of fish (Fig. 2b). Consistently, bioluminescence imaging revealed that incorporation of vaccines with chitosan polymer allowed efficient attachment to mucosal surfaces, as indicated by higher fluorescent intensity than that obtained with naked vaccines (Fig. 2c).

3.3. Protective effect of mucoadhesive vaccines against flavobacterial infection

Vaccinated and control fish were held for 30 and 60 days following vaccination before they were challenged with virulent *F. columnare*. The time course for bath immunization and challenge is shown in Fig. 3a. The fish tolerated the vaccination procedure well. It has been suggested that a positive effect by vaccination is a relative percent survival (RPS) greater than 50% [19]. At 30 days post vaccination with naked and CS-vaccines, the RPS were 4 and 81%, respectively, as shown in Table 1. In this study, mortality in an equivalent group of non-vaccinated and naked vaccine groups were 90% and 87%, respectively versus 17% mortality in the CS-vaccine group. The prolonged protective effect could be also observed in fishes vaccinated with CS-vaccines at 60 days post vaccination (Table 1). 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; haemorrhage, splenomegaly, lesion on the trunk, and/or eroded tail and mouth (Fig. 3c).

4. Discussion

Targeting the vaccine to the particular biological site, where action is needed is difficult. For most aquaculture fish species, immersion vaccines must be directly taken up and processed by appropriate cells of the immune system [20]. Therefore, the use of safe and cheap carriers capable of efficient delivery of antigens to target cells is of importance. In this study, we have successfully developed a mucoadhesive vaccine delivery system to circumvent this problem. We chose *F. columnare*, the causative agent of columnaris disease, as a representative model antigen for this proof-of-concept study.

Table 1
Average percent mortality of tilapia after bath challenge (30 or 60 days post vaccination) with *Flavobacterium columnare*.

Group	Replicate tank	*n*	Average mortality (%)	Average survival (%)	RPS	Remark
Control (30 days)	1	15	90	10	–	–
	2	15				
Naked vaccine (30 days)	1	15	87	13	4	ns
	2	15				
CS-vaccine (30 days)	1	15	17	83	81	**
	2	15				
Control (60 days)	1	15	87	13	–	–
	2	15				
Naked vaccine (60 days)	1	15	97	3	0	ns
	2	15				
CS-vaccine (60 days)	1	15	37	63	58	*
	2	15				

Immersion vaccination for 30 min with 1×10^6 CFU/mL *F. columnare*.
* and ** indicate significant difference compared with control group ($p < 0.05$ using Bonferonni test following one-way ANOVA).

We hypothesized that positive charges of chitosan can enhance the adhesion of inactivated vaccines to negatively charged mucosal membranes, which increases gill accessibility [21]. In our study, mucoadhesive vaccine was prepared by electrostatic coating of inactivated *F. columnare* with a cationic chitosan polymer. Our results showed that the complexation of vaccines with cationic polymers generates positively charged vaccine complexes allowing better adsorption on mucosal surfaces and enhanced protective effect against columnaris disease. A Naked vaccine was used as a control to confirm that they are unable to bind to the mucosal membrane in the absence of chitosan polymers and thus failed to protect fishes from columnaris infection. This enhanced protective effect against infectious diseases may result from the mucoadhesive property of the chitosan polymer. Mucoadhesive polymers increases the contact time with the mucosa [22] thereby increasing the potential of enhancing antigen uptake by the antigen presenting cell. The main mechanism of chitosan mucoadhesion appears to be electrostatic interaction between the positively charged polymer and negatively charged materials such as mucus and cell surface [23–27]. Another possible explanation could be the adjuvant ability of chitosan [28–30]. Chitosan has been extensively investigated for its immunogenic activities, especially via the mucosal routes [31–33]. Therefore, this strategy could be used as an effective method in particular for direct immersion vaccination of fishes.

Despite these promising results, a large range of related clinical parameters need to be measured and monitored over a period of time. Further research should be undertaken in farmed tilapia in order to determine the effectiveness of Mucoadhesive vaccine against *F. columnare*. In addition to related clinical significances, such as Average Daily Gain (ADG), Feed Conversion Ratio (FCR) and survival/mortality rate, *adverse side-effects* (pain and stress) and long-term safety should be evaluated in parallel. The immune response to the vaccine should also be assessed.

5. Conclusion

The vaccine strategy, presented here, is an improved version of a killed vaccine that target the mucosal membrane of tilapia fish. Specifically, we reported on the preparation of mucoadhesive vaccines as well as their physicochemical and biological properties. The analysis of TEM image and zeta-potential also suggested the successful modification of vaccines by chitosan. *In vivo* mucoadhesive study demonstrated the excellent affinity of the chitosan-complexed vaccines toward fish gills as confirmed by bioluminescence imaging, fluorescent microscopy, and spectrophotometric quantitative measurement. Most interestingly, mucoadhesive polymer could increase the efficacy of killed vaccines. Taken together, our study demonstrated the feasibility of mucoadhesive particle as an effective delivery method for a vaccine against infectious *F. columnare* in tilapia by immersion vaccination.

Author contributions

T.Y. was involved in the design and supervision of all experiments. S.K (Sirikorn), K.S., and S.S. were involved in conducting physicochemical experiments. T.Y., N.P., 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. T.Y. and S.K.(Sirikorn) performed the statistical analyses and wrote the manuscript text. All authors reviewed the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

Acknowledgments

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