1 **Chitosan based nanoparticles as protein carriers for**
2 **efficient oral antigen delivery**

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Abstract

This study aimed to investigate the efficacy of nanoparticles based on chitosan as a vehicle for oral antigen delivery in fish vaccination. Carboxymethyl chitosan/chitosan nanoparticles (CMCS/CS-NPs) loaded extracellular products (ECPs) of *Vibrio anguillarum* were successfully developed by ionic gelation method. The prepared ECPs-loaded CMCS/CS-NPs were characterized for various parameters including morphology, particle size (312 ± 7.18 nm), zeta potential ($+17.4 \pm 0.38$ mV), loading efficiency ($57.8 \pm 2.54\%$) and stability under the simulated gastrointestinal (GI) tract conditions in turbot. The *in vitro* profile showed that the cumulative release of ECPs from nanoparticles was higher in pH 7.4 (58%) than in pH 2.0 (37%) and pH 4.5 (29%) after 48 h. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was used as model protein antigen and encapsulated in CMCS/CS-NPs for investigating the biodistribution of antigen after oral delivery to turbot in 24 h. Oral immunization of ECPs-loaded CMCS/CS-NPs group in turbot showed elevated specific antibody and higher concentrations of lysozyme activity and complement activity in fish serum than ECPs solution. CMCS/CS-NPs loaded with ECPs could enhance both adaptive and innate immune responses than the group treated with ECPs solution and suggested to be a potential antigen delivery system.

Keywords: CMCS/CS-NPs; ECPs; Oral immunization; Adaptive and innate immune responses

1. Introduction

Turbot (*Scophthalmus maximus*) is one of the most important commercial fish species worldwide because of their rapid growth, desirable taste and high market value. However, turbot infected by pathogenic bacteria, like *V. anguillarum*, died with a massive mortality, which caused severe economic loss in intensive aquaculture in recent years. *V. anguillarum*, which is commonly found in cultured turbot and wild fish, as well as in bivalves and crustaceans in seawater, caused a fatal vibriosis which was frequently associated with superficial ulcers, haemorrhages at the base of the fins and bloody discharges from the vent [1, 2]. Antibiotics and parasiticides are effective solutions but long term treatment with them would lead to resistance and could be harmful to the environment [3]. Vaccine is regarded as a biologically prepared antigen which contributed to improve the immunity in animals against a particular disease or a group of diseases. Ultimately vaccine is considered as one of the efficient candidates to prevent fish disease by activating host immune response in aquaculture. Current vaccine researches were oriented towards more effective and safer approaches, such as protein and peptide vaccine. Immunization with protein vaccines can elicit very strong and long-lasting innate and adaptive immune responses [4, 5].

The pathogenicity of *V. anguillarum* appeared to involve several extracellular products (ECPs) such as exotoxins, haemolysins [6], as well as outer membrane porin protein [7]. ECPs of *V. anguillarum* were investigated in relation to its virulence and could be regarded as bacterial antigen [8].

At present, oral vaccination was an ideal delivery way in intensive aquaculture for fish farmers, which offered significant advantages over needle-based vaccines, such as

easy handling, low cost of production and being stress free for juvenile fish [3]. Nevertheless, oral vaccination had several defects. For example, the protein vaccine might be degraded in the gastrointestinal (GI) tract due to the low pH and enzymes in the stomach, which lead to the vaccine could not reach the hindgut where antigens were absorbed [3, 9]. Therefore, it is necessary to develop an effective delivery system for oral vaccines. Among these approaches, the biodegradable polymeric nanoparticulate systems were applied as protein and peptide carriers for oral delivery because nanoparticles were able to protect vaccine from degradation and provided controlled-release properties for loaded protein vaccines [10]. In recent years, chitosan (CS) has drawn much attention because of its non-toxicity, biodegradability and excellent biocompatibility, as well as their mucoadhesiveness and permeability-enhancing properties for delivery of peptides, proteins and DNA vaccine [11-13]. Carboxymethyl chitosan (CMCS) is one of the important derivatives which is water soluble and negatively charged in neutral environment. In our previous works, negative charged CMCS was able to form nanoparticles with positive charged CS (CMCS/CS) via electrostatic interaction and maintained the stability of nanostructures in GI tract, which provided a great potential as an oral delivery system for antitumor drugs [14]. In order to develop an effective and easy-to-administer vaccine against vibriosis of fish, CMCS/CS-NPs loading ECPs of *V. anguillarum* were prepared and delivered in turbot through oral route. The immune responses of ECPs-loaded CMCS/CS-NPs were investigated and compared with ECPs solution by detecting specific antibody titers and the activities of lysozyme and complement in fish serum.

2. Materials and methods

2.1. Materials

CS (MW = 400 kDa, degree of deacetylation = 92%) was obtained from Haili Biological Product Co., Ltd. CMCS (MW = 450 kDa, DD = 90%, degree of substitution, DS = 92%) was synthesized and characterized by the method described by Chen [15]. Fluorescein isothiocyanate (FITC) labeled bovine serum albumin (FITC-BSA) was synthesized according to the methods described by Feng [16]. Turbots, juveniles of average weight ranging from 50 to 60 g, were obtained from a local fish farm (Tianyuan, China) and maintained in aerated tanks with sand-filtered seawater. *V. anguillarum* was obtained from marine microbiology laboratory of College of Marine Life Science, Ocean University of China. All other reagents and solvents were of analytical grade.

2.2. Bacterial culture and ECPs preparation

The virulent strain of *V. anguillarum* was routinely maintained in 2216E liquid medium and ECPs of the strain were separated by the method of Rodriguez [17]. The bacteria were shake-incubated at 26 °C for 36 h and then bacterial suspension was centrifuged at 10,000× *g* for 30 min. The supernatant was removed and ECPs were extracted by ammonium sulfate precipitation. The precipitate was redissolved in PBS (pH 7.4) after centrifuging at 10,000× *g* for 20 min at 4 °C then dialyzed for 48 h using a cellulose membrane (Sigma, molecular weight cutoff 8,000-10,000) to remove ammonium sulfate. Protein contents of the ECPs were estimated using the BCA protein assay kits (Solarbio, China).

2.3. Preparation of ECPs loaded CMCS/CS-NPs

ECPs loaded CMCS/CS-NPs were prepared by ionic crosslinking method as previously described with modifications [14]. ECPs dissolved in PBS (1 mg mL⁻¹, pH 7.4) were premixed with CMCS aqueous solution (1 mg mL⁻¹, pH 7.6). Then the mixture was added into CS solution which was dissolved in acetic acid solution (1 mg mL⁻¹, pH 5.5) drop by drop under constant stirring (500 × g). Subsequently, TPP solution (1 mg mL⁻¹) acted as crosslinker was added into the formulation with the volume ratios of 3:4:5:1 (ECPs:CMCS:CS:TPP). The mixture was then shaken at 100 × g for 2 h to form ECPs-loaded CMCS/CS-NPs. The obtained nanoparticles were collected via ultracentrifugation at 12,000 × g for 30 min and then freeze-dried for 48 h. The nanoparticles were re-suspended in PBS (0.2 mM, pH 7.4) before immunization studies. After centrifugation, the amount of ECPs encapsulated in the nanoparticles was determined by measuring the amount of protein remaining in the supernatant by BCA protein assay. The ECPs loading efficacy (LE) and loading capacity (LC) of the nanoparticles were calculated as:

$$\text{LE (\%)} = \frac{\text{Initial antigen conc.} - \text{Unencapsulated antigen conc.}}{\text{Initial antigen conc.}} \times 100$$
$$\text{LC (\%)} = \frac{\text{Initial antigen conc.} - \text{Unencapsulated antigen conc.}}{\text{weight of nanoparticles}} \times 100$$

2.4. Characterization of ECPs loaded CMCS/CS-NPs

2.4.1. Transmission electron microscopy (TEM)

The morphology and size of nanoparticles were observed via a transmission electron microscopy (TEM, JEM-1200EX JEOL Ltd, Japan). Briefly, a drop of sample

suspension was placed onto a carbon-coated copper grid. After 2 min, the drop was taped with a filter paper to remove surface water and then air-dried before observation.

2.4.2. Evaluation of size distribution and zeta potential

The average particle sizes, size distributions, polydispersity index (PDI) and zeta potentials of nanoparticles were measured by using a dynamic laser light scattering technique (Zeta-sizer ZEN3600, Malvern Instruments Ltd, UK) at a detector angle of 90, 670 nm, and 25 °C. The samples were prepared at appropriate concentrations and the solvent was deionized water.

2.5. The stability of nanoparticles in fish simulated gastrointestinal conditions

Oral delivery carriers had several challenges, for example, unstability in the gastrointestinal (GI) tract due to the low pH and enzymes in the stomach, resulting in the low efficacy of protecting the vaccine from degradation. Rodrigues had reported that shortly after feeding, gastro-intestinal pH in Nile tilapia could reach 2.0 and return to 4.5, remaining in this condition until the next feeding [17]. In the hindgut of tilapia, on the other hand, the pH can reach value up to 9.0. Given this, the stability of CMCS/CS-NPs in aqueous solutions simulating fish gastrointestinal conditions was evaluated for samples incubated constantly at 25 °C in deionized water at pH 2.0 and 4.5 (adjusted with HCl), PBS at pH 7.4 and Tris-buffer at pH 9.0 for 2 h , respectively. The CMCS/CS-NPs suspension (1 mL) was placed into a cellulose membrane dialysis tube (molecular weight cutoff 8000-10,000) before it was placed in 49 mL of different simulated fluids and gently shaken in a thermostat shaker bath at $100 \times g$. At predetermined time, the morphology of the nanoparticles was observed by TEM.

2.6. *In vitro* release of ECPs from CMCS/CS-NP

The release of ECPs from nanoparticles was determined as previously described with modifications [18, 19]. *In vitro* release profile of ECPs from the nanoparticles was examined in different release medium (Tris-buffer at pH 2.0, 4.5 and PBS at pH 7.4) for 48 h, respectively and conducted at 25 ± 1 °C to maintain the corporal temperature of the fish. 1 mL ECPs:CMCS/CS-NPs (1 mg mL^{-1}) were placed into Eppendorf tubes with 1 mL of different release medium at $100 \times g$ in a shaking incubator. At designated time intervals, 1 mL of the medium was taken for BCA protein measurement, and fresh medium of the same volume was added to the incubator. The protein content of the supernatant was measured in triplicate.

2.7. *Molecular weight integrity of the model protein antigen BSA released from nanoparticles*

During the loading of vaccine into the nanoparticles, the vaccine's structural stability is essential for retaining its immune activity [18]. Changes of molecular weight would indirectly reflect the disruption of spatial structure, which can lead to the loss of protein activity. In the present study, BSA used as model protein antigen was loaded into CMCS/CS-NPs by ionic crosslinking method and analyzed the molecular weight integrity of BSA released from the nanoparticles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with non-encapsulated proteins as control.

2.8. *Biodistribution of FITC-BSA in vivo*

Turbots were acclimatized in the aquarium for 15 days prior to the start of the

experiment. In order to investigate the biodistribution of antigen in turbot, model protein BSA labeled with FITC was encapsulated into CMCS/CS-NPs by ionic gelation instead of ECPs of *V. anguillarum* for the reason that ECPs were a mixture of products with low labeling efficiency. Different formulations (0.5 mL FITC-BSA loaded CMCS/CS-NPs, FITC-BSA solution and PBS) were orally administered to turbot by using a catheter. After 24 h, intestine, liver, kidney and spleen were excised, made into frozen section, and viewed under a fluorescence microscope.

2.9. Immunization protocol

The turbot were maintained under standard pathogen-free conditions and provided with free access to fish feed during the experiment. After acclimation for 15 days, the healthy turbot were separated into 3 groups, 15 fishes in each group were orally administrated separately by feed syringe with different formulations: ECPs solutions (containing 25 µg ECPs per gram of fish, 0.5 mL), CMCS/CS-NPs, (equivalent to 25 µg ECPs per gram of fish, 0.5 mL) whereas control group were orally delivered with 0.5 mL PBS (0.2 mM, pH 7.4) only. All groups received vaccines on day 0, 7 and 21. To assess immune responses, fish were sampled at days 7, 21 and 35 (prior to the first immunization) (Fig. 1). Before experiment, live turbot were kept on ice for 2 min then the blood samples were collected from the tail veins and kept at 4 °C for 4 h followed by centrifuging for 10 min at 3000 × g. Next, the serum was collected and stored at -70 °C until analysis for specific antibody and innate parameters.

Fig. 1. Schematic representation of the oral immunization protocol.

2.10. *In vivo immune responses*

2.10.1. *Specific antibody (IgM) detection by enzyme-linked immunosorbent assay (ELISA)*

Specific antibody in fish serum was determined by enzyme-linked immunosorbent assay (ELISA) which was performed using 96 well microtiter polystyrene plates. 100 μ L ECPs ($20 \mu\text{g mL}^{-1}$) dissolved in carbonate-bicarbonate solution (pH 9.6) was coated with plate overnight at 4 °C. The plate was washed thrice in PBS with 0.05% Tween-20 (PBST) and blocked with 5% skim milk powder for 2 h at 22 °C. After blocking, the wells were further washed with PBST. The fish sera raised against various antigenic treatments were 2-fold serially diluted with PBS (pH 7.4) ranging from 2 to 2^{12} and then added into antigen-coated wells in duplicate. The plates were incubated for 3 h at 22 °C and washed thrice in PBST. Then the mouse-anti-turbot IgM (Aquatic diagnostics Ltd., UK) was added (1 h incubation) followed by goat-anti-mouse IgG conjugated to HRP (1 h incubation). The plate was then thoroughly washed with PBST and the reaction was visualized after adding 100 μ L well⁻¹ of TMB/H₂O₂. The action was allowed to proceed for 10 min at 22 °C, stopped with 50 μ L well⁻¹ of 2M sulphuric acid (H₂SO₄) solution, and absorbance was read at 450 nm with an automatic plate

reader. Antibody titer was calculated as follows:

$$P/N = OD_{450 \text{ vaccinated}} - OD_{450 \text{ non-vaccinated}}$$

When P/N value was greater than 2.1, antibody titer is equal to the highest dilution multiple.

2.10.2. Innate immuneresponse

Innate immune system plays an essential role in the early defense against pathogen infection. Lysozymes can function as an innate opsonin to some extent or as a lytic enzyme [20]. The complement system is a vital part of innate immune defense against foreign organisms and is responsible for various immune effector functions including elimination of invading pathogens, promotion of inflammatory responses, in addition to modulation of adaptive immune response [3, 21]. Lysozyme activity and complement activity in fish serum were detected by ELISA kit. According to the specification, the microtiter plates were coated by purified lysozyme and complement, respectively, to make solid-phase antibody. The sera of turbot were diluted with sample diluent and added into 96-well microtiter plates. With HRP labeled antibody, the antibody-antigen-enzyme labelled antibody complex was prepared, followed by adding TMB substrate solution to the wells. The reaction was terminated by the addition of H₂SO₄ solution and absorbance was read at 450 nm. On the basis of the standard curve, the concentrations of lysozyme activity and complement activity were calculated.

2.11. Statistical analysis

The assays were performed at least in triplicate on separate occasions. All data were presented as mean $\pm$ standard deviation (SD). A statistical analysis was performed using

one-way ANOVA for data. $p < 0.05$ was taken to indicate statistical significance.

3 Results and discussion

3.1. Preparation and characterization of ECPs loaded CMCS/CS-NPs

ECPs loaded CMCS/CS-NPs were prepared by the ionic gelation among positively charged CS and negatively charged CMCS. TPP was used as a cross-linker to further stabilize nanoparticles structure (Fig. 2). As is reported, various parameters: size, physicochemical properties, encapsulation efficiency are known to play a key function in designing efficient polymeric nano/micro particles based delivery system through molecular interaction [22, 23].

Fig. 2. Schematic illustrations of the preparation process of ECPs-loaded CMCS/CS-NPs by ionic gelation method.

The morphological characterizations of CMCS/CS-NPs and ECPs loaded CMCS/CS-NPs were evaluated by a transmission electron microscope (Fig. 3). The nanoparticles were found to be spherical, smooth, non-aggregated with mean sizes of 255 ± 6.27 nm and 312 ± 7.18 nm, respectively, which indicated that the particle size increased when successfully loaded ECPs into nanoparticles. It has been proposed that the size of nanoparticles plays a key role in their adhesion to and interaction with the

biological cells. The possible mechanism for the particles with a diameter of 50-500 nm to pass through the gastrointestinal (and other physiological) barriers is by endocytotic uptake [24, 25]. The zeta potential values of the nanoparticles were $+16.4 \pm 0.51$ mV and $+17.4 \pm 0.38$ mV, respectively, which was probably due to that some CS molecules were entangled onto the surfaces of particles, producing a positive surface charge. Likewise, absolute values of zeta potential were greater than 16 mV, which indicated that the prepared nanoparticle system was stable. The polydispersity index (PDI) was a measurement for distribution of NPs and gave a distribution range from 0.00 to 0.50. The value of PDI more than 0.5 indicated the aggregation of particles [26]. The PDI value of CMCS/CS-NPs (0.19 ± 0.02) and ECPs loaded CMCS/CS-NPs (0.27 ± 0.03) confirmed its monodisperse nature. Encapsulation of protein antigens depends on many factors like polymer nature, molecular interaction between polymers, method of formulations [23]. ECPs-loaded CMCS/CS-NPs were generated by electrostatic interaction and the loading content and loading efficiency of ECPs were $14.5 \pm 0.31\%$ and $57.8 \pm 2.54\%$, respectively (Table 1).

Table 1

Mean particle size, zeta potential and PDI of CMCS/CS-NPs and ECPs-loaded CMCS/CS-NPs. The results were expressed as mean $\pm$ SD ($n = 3$)

Formulations	Mean particle size (nm)	PDI	Zeta potential (mV)	LC (%)	LE (%)
CMCS/CS-NPs	255 ± 6.27	0.19 ± 0.02	$+16.4 \pm 0.51$	-	-
ECPs-loaded CMCS/CS-NPs	312 ± 7.18	0.27 ± 0.03	$+17.4 \pm 0.38$	14.5 ± 0.31	57.8 ± 2.54

Fig.3. TEM images and size distribution by intensity of CMCS/CS-NPs (a, c) and ECPs-loaded CMCS/CS-NPs (b, d). Scale bar 500 nm.

3.2. The stability of nanoparticles in simulated gastrointestinal conditions of fish

Stability in GI tract, high initial burst and structural integrity of the encapsulated proteins are the factors which should be focused to be avoided when developed an oral delivery carrier. The stability of CMCS/CS-NPs was performed in simulated gastrointestinal conditions at pH 2.0, 4.5, 7.4 and 9.0 every 2 h, respectively. The morphology of nanoparticles in different pH was evaluated by TEM. Feng has explained the pH responsibility of CMCS/CS-NPs is closely related to the protonation and deprotonation of CS and CMCS in different pH [14]. As shown in Fig.4, in the first two hours (pH 2.0), CMCS/CS-NPs presented a relatively complete structure with a spherical shape, which did not show significant morphology variation. And then, after two hours incubation in pH 4.5, the complex structures were a little loose and started to lose spherical shape. The reason was that in pH 2.0 and 4.5, the amino groups (pK_a 6.5)

on CS and CMCS were protonized, phosphate groups (pK_a 1.0-2.1) on TPP were ionized, and carboxyl groups (pK_a 2.0-4.0) on CMC were partially or completely disassociated. Therefore, positively charged CS and negatively charged CMCS, TPP were able to form polyelectrolyte complexes via electrostatic interaction, giving rise to spherical particles (Fig. 4). In PBS (pH 7.4), the nanoparticles aggregated together and became gradually loose. Finally, CMCS/CS-NPs almost lost spherical shape and became disintegrated after two hours incubation in pH 9.0 which simulated the environment of hindgut in fish. In basic environment (pH 7.4, pH 9.0), the amino groups on CS and CMCS partially or completely deprotonized and the CMCS/CS-NPs disintegrated and precipitated.

Fig. 4. TEM images of CMCS/CS-NPs at successive different pH 2.0 (a), 4.5 (b), 7.4 (c) and 9.0 (d) for 2h, respectively. Scale bar 500 nm.

3.3. *In vitro* release

The release rate of ECPs from the CMCS/CS-NPs was measured in Tris-buffer (pH 2.0, 4.5) and PBS (pH 7.4) at 25 °C for 48 h (Fig. 5). ECPs released from

CMCS/CS-NPs in Tris-buffer and PBS were typified by an initial rapid release when almost 28% (pH 2.0), 21% (pH 4.5) and 48% (pH 7.4) of the protein were released within the first 4 h followed by a slow and much reduced further release lasting for 2 days. In addition, the highest cumulative release of ECPs was 58% at pH 7.4, followed by 37% at pH 2.0 and 29% at pH 4.5 after 48 h. At the beginning (< 4 h), the release of ECPs was fast and then it was released very slowly, suggesting that the nanoparticles might act as a barrier against the burst release of entrapped ECPs. The cumulative release of ECPs is a little higher in pH 2.0 than that in pH 4.5 with the possible reason that at pH 2.0, the protonated carboxyl groups on CMCS weakened the electrostatic interaction among CS, CMCS and TPP and thus the stability of nanoparticles weakened.

Fig. 5. ECPs release profiles from the CMCS/CS-NPs at different incubation time points at pH 2.0, 4.5 and 7.4. Data represented the mean $\pm$ SD (n = 3).

3.4. Molecular weight integrity of the model protein antigen released from nanoparticles

Overall, the low immune potency of the oral protein vaccine might be due to the

challenge including the structural instability of protein vaccine during processing and encapsulation. In the present study, the model protein BSA was encapsulated into nanoparticles using ionic gelation and evaluated the molecular weight integrity of BSA released from CMCS/CS-NPs when incubated in PBS at 37 °C by SDS-PAGE (Fig. 6). The BSA released from CMCS/CS-NPs was suspended in loading buffer and heated for 3 min at 100 °C just before SDS-PAGE. The suspension was run on a gradient gel and then tested for protein degradation by coomassie staining. As shown in Fig. 6, the molecular weight of BSA released from the CMCS/CS-NPs was similar to native BSA without apparent changes, indicating that the molecular weight of BSA was not altered during the loading of the BSA into the nanoparticles, which was in agreement with the view of Li [19].

Fig. 6. Evaluation of the integrity of molecular weight of BSA released from the nanoparticles by SDS-PAGE. Lane 1: native BSA, Lane 2: BSA released from CMCS/CS-NPs, Lane 3: marker.

3.5. Biodistribution of FITC-BSA in turbot

To determine the biodistribution of antigen in tissues, FITC labeled BSA loaded CMCS/CS-NPs were orally administered to turbot. Organs were excised at 24 h after oral administration of different formulations to turbot. As shown in Fig.7, the detectability of the FITC-BSA within kidney, spleen, intestine and liver was due to the fact that fish have a lymphatic system including kidney, spleen and mucosa-associated lymphoid tissue. While there were no obvious green signals in the PBS group, the group of FITC-BSA:CMCS/CS-NPs showed significant green signals compared with FITC-BSA group in different tissues. The figure suggested that CMCS/CS-NPs could protect antigenic protein to efficiently enter the spleen and kidney, which are critical for mounting an immune response. It has been reported chitosan and its derivatives have the properties of mucoadhesiveness and permeation enhancing effect which makes them suitable for oral delivery [27-29]. In the previous studies, chitosan-based nanoparticles were able to sustain at a high level in blood and evidenced the capability of penetrating into the tissues [30, 31].

Fig. 7. Fluorescence images of tissues slices of turbot. Scale bar 100 μm .

3.6. Serum antibody responses

After oral vaccination of turbot 3 times, the antibody titers in fish serum orally immunized with ECPs-loaded CMCS/CS-NPs, ECPs solution and PBS were shown in Fig. 8. ECPs-loaded CMCS/CS-NPs treated group showed comparatively higher antibody titers than ECPs solution and PBS groups. Moreover, the highest antibody titer was observed in ECPs-loaded CMCS/CS-NPs treated group at 35 days post the vaccination and had reached 7 ($p < 0.05$), indicating that CMCS/CS nanoparticles could effectively protect the vaccine from degradation when passing through the fish GI tract and delivered ECPs to transport across the intestinal epithelium to induce adaptive immune response. Maybe the reason was in relation with the positively charged ECPs:

CMCS/CS-NPs directed to the natively mucus, which prolonged residence time on it [10]. By contrast, a little increase of antibody titers was detected in turbot orally vaccinated with ECPs solution. At the same time, there was no obvious change of antibody titers in serum of control group.

Fig. 8. Serum specific antibody (IgM) titers in turbot immunized with ECPs-loaded CMCS/CS-NPs, ECPs solution and PBS. Data represented the mean $\pm$ SD (n = 5). * $p < 0.05$ as compared with control.

3.7. Lysozyme and complement evaluation

In fish, the nonspecific immunity is regarded as an essential component to produce first line defense against pathogens due to some limitations of adaptive immune system, the limited repertoire of antibodies, the slow proliferation and memory of their lymphocytes [19], so the activities of lysozyme and complement have a relative fast immune response. Both the lysozyme activity (Fig. 9a) and complement activity (Fig. 9b) in fish serum were monitored for ECPs loaded CMCS/CS-NPs along with ECPs solution and PBS groups. The order of the lysozyme activity in serum orally administrated with different groups is as follows: CMCS/CS-NPs > ECPs solution > control group. The activity of lysozyme was significantly higher ($p < 0.05$) in

CMCS/CS-NPs group than the control group at 21 days post-the first immunization. The similar trend was found for the complement activity which was significantly higher ($p < 0.05$) than that treated with PBS at 21 days post-immunization when treated with CMCS/CS-NPs in turbot (Fig. 9b). The above results were in agreement with the use of CMCS/CS-NPs as an antigen carrier in fish and its application in orally antigen delivery to induce immune responses.

Fig. 9. The lysozyme activity (a) and complement activity (b) of serum in different groups at 7, 21 and 35 days post-immunization. Results were achieved from 5 or more repeated samples and presented as mean $\pm$ standard deviation (SD). * $p < 0.05$ as compared with control.

4 Conclusion

In the present work, CMCS/CS-NPs as oral carriers were prepared to encapsulate ECPs of *V. anguillarum* for oral vaccination in turbot. Besides spherical morphology, uniform size and high loading efficiency, CMCS/CS-NPs presented favorable pH responsive stability in GI tract, which could effectively protect antigen from degradation

in the stomach and had sustained release of ECPs in the intestine. The biodistribution of FITC-BSA in turbot also proved CMCS/CS-NPs could protect antigenic protein to enter the spleen and kidney, which were critical for mounting an immune response. Thus, these results provided evidences of CMCS/CS-NPs as promising nanocarriers for oral vaccine delivery, partly contributing to enhancing both adaptive and innate immune responses in fish.

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