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# Chitosan based nanoparticles as protein carriers for 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 ± 0.38 mV), loading efficiency (57.8 ± 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.



## 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 *Vibrio 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

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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 8000–10,000) 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 ultra centrifugation 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:

$$\mathrm{LE}(\%) = \frac{\text{Initial antigen conc.} - \text{Unencapsulated antigen conc.}}{\text{Initial antigen conc.}} \times 100$$

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

### 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*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*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.