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*Research Article*

# Biocompatible fish-targeted chitosan-TPP/CaP hybrid nanocarrier (Cs/Tpp-Cap) for oral DNA vaccine delivery and immune gene expression in goldfish

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**Abstract**

This study evaluates the potential of chitosan tripolyphosphate/calcium phosphate (Cs/Tpp-Cap) nanoparticles as carriers for oral DNA vaccine delivery in fish. In aqueous solution, positively charged Cs/Tpp-Cap nanoparticles interact electrostatically with negatively charged DNA molecules, resulting in the spontaneous self-assembly of stable nanocomplexes. This self-assembly strategy provides an innovative means of modifying anionic Cs/Tpp-Cap hybrid nanoparticles for effective oral DNA vaccine applications. The DNA vaccine was constructed by cloning the *Vibrio anguillarum* outer membrane porin (OMP) gene (993 bp) into the eukaryotic expression vector pcDNA3.1, generating the recombinant plasmid pVAOMP DNA. The morphology and structural characteristics of Cs/Tpp-Cap nanoparticles were characterized using scanning electron microscopy (SEM), high-resolution transmission electron microscopy (TEM), and Fourier-transform infrared spectroscopy (FTIR). For encapsulation, equal volumes of heated Cs/Tpp-Cap and pVAOMP DNA solutions were combined, achieving a DNA binding efficiency of 85%. Gene distribution and expression analyses confirmed the successful incorporation of the *VAOMP* gene and revealed the upregulation of immune-related genes in vaccinated fish. These findings demonstrate that Cs/Tpp-Cap nanoparticles can function as an efficient, biocompatible, and non-viral delivery system for oral DNA vaccination in aquaculture species, providing a promising platform for disease prevention in fish farming.



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## Introduction

Nanotechnology has emerged as a promising platform for the delivery of biological macromolecules, including vaccine antigens, for disease prevention and control. Calcium phosphate, Chitosan tripolyphosphate, calcium–alginate and Poly (lactic-co-glycolic acid) are biodegradable polymeric nanoparticles developed as a new technique for vaccine delivery (Roy *et al.,* 2003; Tian and Yu, 2011; Vimal *et al.,* 2014; Nait Mohamed and Laraba-Djebari, 2016; Thirumalaikumar *et al.,* 2023). A vaccine is a biologically produced antigen that strengthens an animal's defences against a single or a class of diseases. To protect fish from various bacterial and viral diseases, several vaccine types have been tried, including DNA vaccines, recombinant protein vaccines, and formalin- or heat-killed whole cell vaccines (Boesen *et al.,* 1997; Yuasa *et al.,* 2002; Sommerset *et al.,* 2003, 2005). The goal of current research on vaccines is to replace traditional vaccinations with safer, more effective alternatives like DNA vaccines. Recently, a number of innovative techniques have been developed to introduce foreign DNA into cells. One tactic is a non-viral delivery system (Abourehab *et al.,* 2022; Radhakrishnan *et al.,* 2023; Palaniappan *et al.,* 2025).

Previously reported pSVbGal plasmid DNA loaded calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery in mice (Roy *et al.,* 2003). Although a variety of cell types have shown high transfection efficiency with viral gene delivery systems, these systems also have some drawbacks, including oncogenic effects and inflammatory responses are induced virtually (Engelhardt *et al.,* 1993). To overcome these problems, non-viral gene delivery systems using calcium phosphate nanoparticles have been developed to deliver genes in *in vivo* and *in vitro* studies (Bisht *et al.,* 2005; Sokolova *et al.,* 2006). Germershaus *et al*. (2008) and Chua *et al*. (2012) have developed chitosan based nanoparticles (trimethyl chitosan, polyethylene glycol-graft-trimethyl chitosan and chitosan microparticles) for gene and vaccine delivery applications.

Vibriosis is one of the major bacterial diseases affecting fish, caused by species of the family *Vibrionaceae* such as *Vibrio anguillarum*, *Vibrio vulnificus*, and *Vibrio harveyi*. This disease results in substantial economic losses for the global aquaculture industry (Hegde *et al.,* 2023). In India, *Lates calcarifer* (Asian sea bass) is an economically important species widely cultured in aquaculture. However, *Vibrio anguillarum* infection represents a major challenge in sea bass farming, both in India and globally.

Bisht *et al*. (2005) have reported the delivery of plasmid DNA (pDNA) using calcium phosphate nanoparticles in HeLa cell lines. Similarly, anionic hybrid nanoparticles composed of PEGylated carboxymethyl chitosan/calcium phosphate have been employed for siRNA delivery in anticancer therapy (Xie *et al.,* 2014). The Chitosan/TPP (CS/TPP) nanoparticles have also been evaluated as gene delivery nanocarriers, where the ionic gelation technique was adapted to encapsulate various nucleic acids, including plasmid DNA (Thirumalaikumar *et al.,* 2021).

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In the present study, an innovative approach was undertaken by utilizing chitosan tripolyphosphate/calcium phosphate (Cs/Tpp-Cap) hybrid nanoparticles, owing to their favorable biocompatibility and biodegradability. These Cs/Tpp-Cap nanoparticles were employed to deliver the pVAOMP DNA vaccine in fish via the oral route.

## Materials and methods

### *Materials*

Low molecular weight chitosan (CS) and tripolyphosphate (TPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DNase I and RNase A enzymes were obtained from Promega (Madison, WI, USA). Ethanol and acetic acid were sourced from Hi-Media Laboratories (Mumbai, India). Other chemicals, including trisodium citrate, sodium pyrophosphate, dibasic sodium phosphate, and calcium chloride, were purchased from SRL (Chennai, India).

Mass preparation of the plasmid DNA (pVAOMP) was carried out in-house. *Escherichia coli* DH5α strains harboring the plasmid were cultured and processed using the alkaline lysis method described by Sambrook *et al*. (1989), with triple-distilled water used throughout.

### *Experimental animal and rearing systems*

Healthy *Carassius auratus* (goldfish) with an average body weight of 8±1.2 g were obtained from a private commercial hatchery in Chennai, Tamil Nadu, India. The fish were maintained in 500 L fiberglass tanks with continuous aeration at a water temperature of 25–28°C and acclimatized to laboratory conditions prior to experimentation. During both the acclimatization and experimental phases, fish were fed orally twice daily with commercial fish feed. After acclimation, fish were distributed into separate experimental tanks. Water quality parameters were monitored regularly and maintained as follows: dissolved oxygen, 5.22 ± 0.67 mg $L^{-1}$; pH, 7.5±0.45; nitrites, 0.014±0.05 mg $L^{-1}$; and ammonia, 0.112±0.05 mg $L^{-1}$ throughout the study.

### *Construction and preparation of plasmid (pVAOMP) DNA vaccine*

The outer membrane porin (OMP) gene of *Vibrio anguillarum* was amplified from genomic DNA using polymerase chain reaction (PCR) with a gene-specific primer set. The identity of the amplified PCR product was confirmed by sequencing. A detailed protocol for the construction and preparation of the DNA vaccine has been described in our previous publication (Vimal *et al.,* 2012b).

### *Preparation of chitosan/tripolyphosphate (Cs/Tpp) and calcium phosphate (Cap) nanoparticles*

Preparation of chitosan/tripolyphosphate (Cs/Tpp) nanoparticles has been described in our earlier publication (Vimal *et al.,* 2012b; Thirumalaikumar *et al.,* 2021). Calcium phosphate nanoparticles (Cap) were synthesized following the procedure described by Ramachandran *et al*. (2009) with slight modification. Briefly, 100 mL of 15.625 mM trisodium citrate was mixed with 500 mL of 12.5 mM calcium chloride and stirred for 15 min. The pH of the medium was adjusted to 10 by the addition of 25 mM sodium pyrophosphate.

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Subsequently, 500 mL of 12.5 mM dibasic sodium phosphate solution was added dropwise to the reaction mixture under continuous stirring at 300 rpm. The resulting suspension was kept overnight to allow the reaction to proceed to completion. The nanoparticle suspension was then washed, sonicated for 5 min at 50 W, and freeze-dried for 16 h at –20°C. Finally, the dried product was lyophilized at –40°C.

*Preparation and characterization of Cs/Tpp-Cap nanoparticles*

An equal volume of chitosan tripolyphosphate solution and calcium phosphate solutions were mixed which was then vortexed for 30 seconds. The resulting suspension was transferred into centrifuge tubes and centrifuged at 15,000 rpm for 30 min at 10°C to separate the nanoparticles.

The morphology of the Cs/Tpp-Cap nanoparticles was observed by SEM (Scanning electron microscopy) equipped with a backscattered electron detector at 10kV and high-resolution transmission electron microscopy (TEM) equipped with a backscattered electron detector at 200 kV. The degree of cross-linking between Cs/Tpp and Cap was evaluated by Fourier transform infrared spectroscopy (FTIR). FTIR spectra were recorded at a resolution of 4 $cm^{-1}$ using KBr pellets (1:150 ratio) on a Bruker spectrometer. Absorbance scans were analyzed for variations in peak intensity between 500 and 4000 $cm^{-1}$.

*Encapsulation efficiency of pVAOMP DNA in Cs/Tpp-Cap*

The pVAOMP DNA vaccine (100 µg/mL) and Cs/Tpp-CaP nanoparticles (3 mg/mL, pH 5.5) were separately heated to 55 °C. Following the protocol of Bozkir and Saka (2004), equal volumes of the heated Cs/Tpp-CaP and pVAOMP DNA solutions were mixed and vortexed at 2500 rpm for 30 s. The mixture was carefully transferred into centrifuge tubes and centrifuged at 14,000 rpm for 30 min at 10 °C to separate the DNA-loaded nanoparticles. The supernatant was collected, and the concentration of unbound pVAOMP DNA was quantified using a UV spectrophotometer at 260 nm. The encapsulation efficiency (EE) was calculated according to the formula described by Gan and Wang (2007):

$$EE = \frac{\text{Total amount of pVAOMP DNA} - \text{Free amount of pVAOMP DNA in supernatant}}{\text{Total amount of pVAOMP DNA}} x100$$

*Preparation of experimental fish feed*

The formulation of the basal diets was prepared using the method described by Thirumalaikumar *et al*. (2021) as shown in Table 1. All dry ingredients were powdered, passed through a 650 µm sieve and mixed with water until very soft biscuit-textured dough was obtained. Then the mixture was steam sterilized (autoclaved at 120°C for 20 min). After cooling, 10 g (wet weight) of the fish feed paste was combined with the lyophilized DNA–Cs/Tpp-Cap complex preparation, which contained approximately 3 mg of Cs/Tpp-Cap and 100 µg/mL of the DNA construct. The mixture was thoroughly homogenized and pelletized manually to obtain feed pellets of 1.5 mm size. The

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pellets were dried in a hot air oven at 50°C for 2 h before use.

**Table 1: Formulation of the experimental diet (g %).**

| Ingredients | Basal diet (g%) |
|---|---|
| Fish meal | 14 |
| Ragi flour | 15 |
| Wheat flour | 14 |
| Corn flour | 17 |
| Rice bran | 10 |
| Groundnut oil cake | 18 |
| Tapioca powder | 5 |
| Fish oil | 5 |
| Vitamin premix[a] | 1 |
| Mineral premix[b] | 1 |

[a] Vitamin (IU or mg per kg premix): Vitamin A, 700000IU; Cobalamin, 200IU; Vitamin D3, 70000IU; Vitamin E, 250 mg; Nicotinamide, 1 g.

[b] Mineral premix (per kg premix): Cobalt, 150 g; Copper, 1200 mg; Iodine, 325 mg; Iron, 1500 mg; Magnesium, 6000 mg; Manganese, 1500 mg; Potassium100 mg; Sodium, 5.9 mg; Sulphur, 0.72%; Zinc, 9600 mg; Calcium, 25.5%; Phosphorus, 12.75%.

*Oral delivery of Cs/Tpp-Cap loaded pVAOMP DNA into fish*

Goldfish were used in the pVAOMP DNA vaccine delivery study and divided into three groups at a density of 10 fish per tank, with three replicate tanks per group (30 fish per group in total). Group I fish were fed flakes without Cs/Tpp-CaP (control), Group II fish received flakes containing pcDNA3.1 nanoformulated with Cs/Tpp-CaP (vector control), and Group III fish were fed flakes containing the pVAOMP DNA vaccine nanoformulated with Cs/Tpp-CaP at a concentration of 100 µg/mL plasmid complex. All groups were fed their respective experimental diets twice daily at a rate of 3% of initial body weight for 10 days. At the end of the feeding trial, fish were dissected, and tissues including the heart, liver, intestine, muscle, and gills were aseptically collected for downstream analyses by PCR, ELISA, and RT-PCR.

*Tissue distribution of pVAOMP DNA by PCR*

PCR was used to examine the tissue distribution of pVAOMP DNA in fish feed containing Cs/Tpp-Cap nanoformulated pVAOMP DNA. DNA was extracted from heart, liver, intestine, muscle, and gill samples using the protocol described by Lo *et al*. (1996). The presence of *V. anguillarum* pVAOMP DNA was confirmed following the PCR protocol outlined by Vimal *et al*. (2012b).

*RNA isolation and immune gene expression analysis*

RT-PCR was performed for transcriptional analysis of each tissue sample. Following the manufacturer's instructions, total RNA was extracted from each sample (~50–100 mg) using Trizol reagent (Invitrogen, Carlsbad, USA). 1 µg of mRNA was utilized for synthesis of cDNA using the RevertAid cDNA synthesis kit (Thermo Fisher Scientific, USA). The qPCR reaction was performed by Applied Biosystems Steponeplus (California, USA) as we described earlier (Thirumalaikumar *et al.,* 2023). The relative expression levels of immune-related genes were normalized against the housekeeping gene β-actin (Livak and Schmittgen 2001).

*Enzyme-linked immunosorbent assay (ELISA)*

ELISA was performed to confirm VAOMP gene expression in different fish tissues using an antiserum raised against the outer

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membrane protein of *Vibrio anguillarum*. Tissue samples from fish fed with Cs/Tpp-CaP–nanoformulated pVAOMP DNA were placed in the wells of a flat-bottomed ELISA plate and incubated overnight at 4 °C. The plates were then washed thoroughly with PBS and blocked for 1 h at 37°C with 2% BSA in PBS. After washing with PBS/T, the plates were incubated for 2 h at 37°C with antiserum raised against VAOMP. Following incubation, the plates were rinsed three times with PBS/T and PBS (2 min each) and incubated for 1 h with 100 µL of rabbit anti-mouse IgG conjugated to alkaline phosphatase. After another series of washes (three times each with PBS/T and PBS, 2 min each), 0.5 mg/mL alkaline p-nitrophenyl phosphate substrate solution was added. The development of a yellow color, corresponding to the enzyme–substrate reaction, was measured at 405 nm using an automated ELISA plate reader (Labsystems, USA).

## Result

*Characterization of Cs/TPP-Cap nanoparticles*

FTIR spectroscopy confirmed the structural integrity and successful preparation of the Cs/TPP-CaP hybrid nanocarriers (Fig. 1).

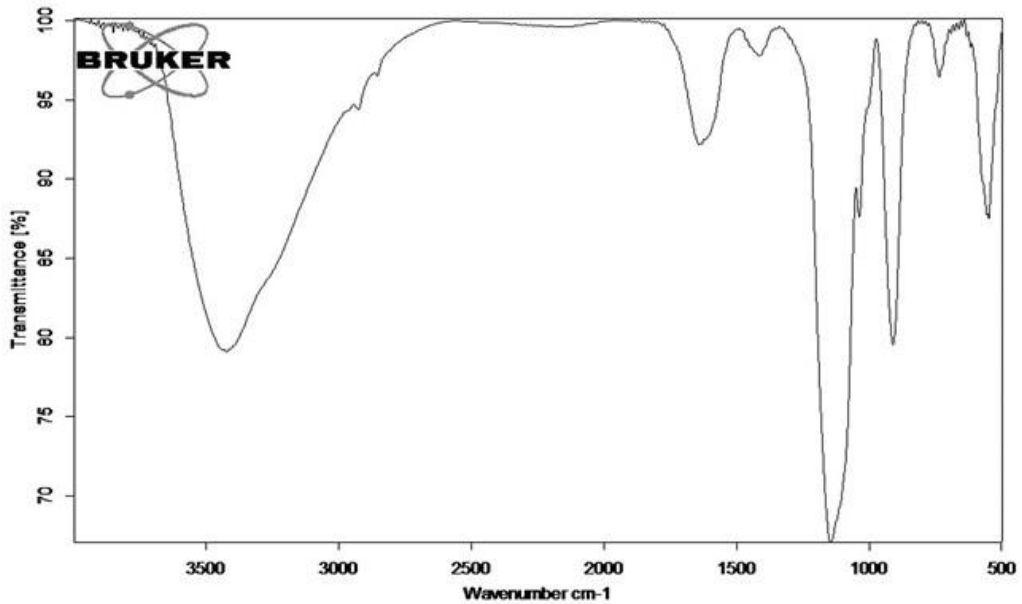



**Figure 1:FTIR spectra of Chitosan tripolyphosphate/calcium phosphate nanoparticles (Cs/Tpp-Cap).**

A broad absorption peak around 3400 $cm^{-1}$ indicated overlapping O–H and N–H stretching vibrations of chitosan, while characteristic amide I (~1650 $cm^{-1}$) and amide II (~1550 $cm^{-1}$) peaks further supported the chitosan backbone. Absorption bands in the 1250–1300 $cm^{-1}$ region (P=O stretching) and at ~1050 $cm^{-1}$ (asymmetric P–O–P stretching) confirmed the presence of TPP, and additional peaks below 1000 $cm^{-1}$ indicated phosphate ion vibrations, validating the incorporation of calcium phosphate. Collectively, these findings verify the formation of a stable Cs/TPP-CaP polyelectrolyte complex, reinforcing its suitability as a nanocarrier for DNA vaccine delivery. The size and morphology of Cs/Tpp-Cap nanoparticles

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were characterized using SEM and TEM, as shown in Figures 2 and 3, respectively. SEM analysis revealed that the nanoparticles were spherical in shape, self-aggregated, and displayed a relatively uniform particle size distribution, ranging from 50 to 80 nm (Fig. 2). TEM analysis further confirmed these observations, showing nanoparticles with a consistent spherical morphology and uniform structure, with sizes also falling within the 50–80 nm range (Fig. 3). These results demonstrate that the Cs/Tpp-Cap nanoparticles were successfully synthesized with nanoscale dimensions and homogenous morphology.

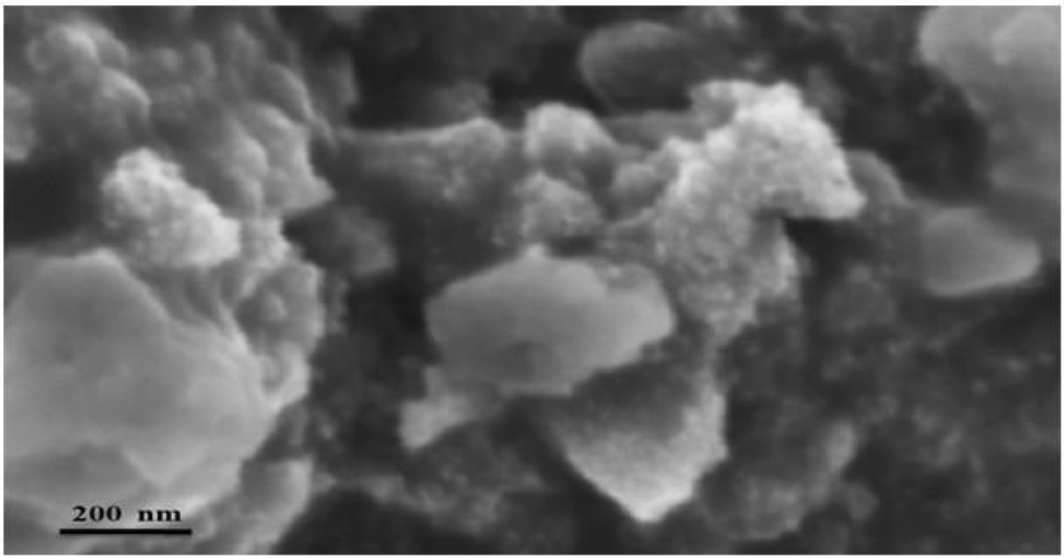



**Figure 2: SEM images of Chitosan tripolyphosphate/calcium phosphate nanoparticles (Cs/Tpp-Cap).**

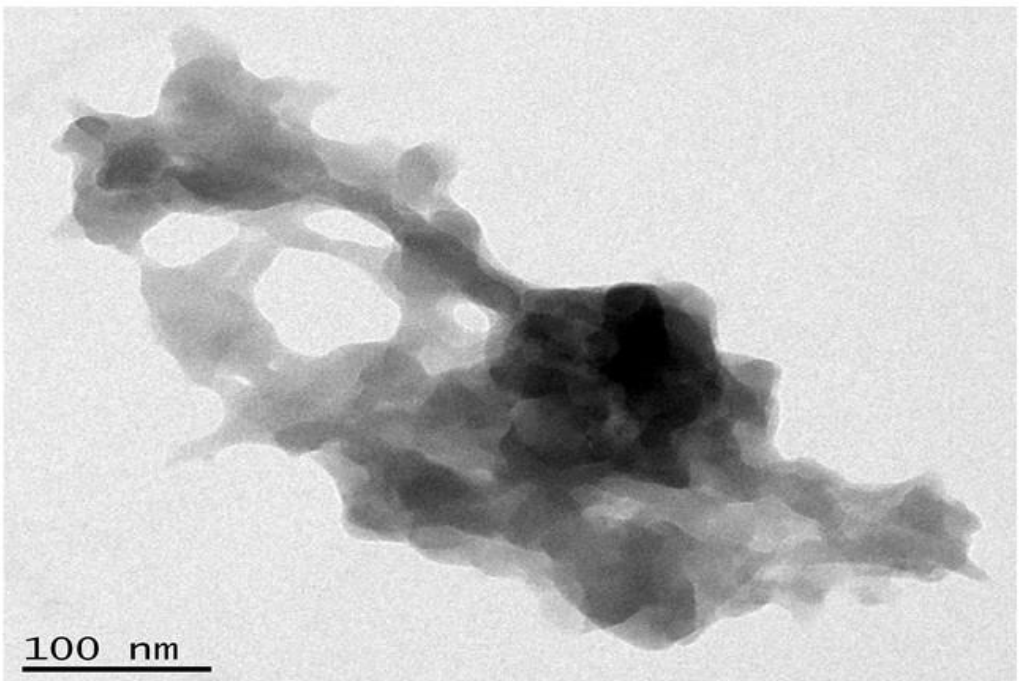



**Figure 3: TEM images of Chitosan tripolyphosphate/calcium phosphate nanoparticles (Cs/Tpp-Cap).**

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*Encapsulation efficiency of pVAOMP DNA*

The encapsulation efficiency of pVAOMP DNA within Cs/Tpp-CaP nanoparticles was determined to be 85%, indicating a high DNA-binding capacity and effective loading of the plasmid into the nanocarrier system. This result highlights the suitability of Cs/Tpp-CaP nanoparticles for stable and efficient DNA vaccine delivery.

*Tissue distribution of pVAOMP DNA*

After 10 days of feeding, PCR analysis confirmed the distribution of pVAOMP DNA in various tissues of fish administered Cs/Tpp-CaP–pVAOMP DNA (Fig. 4). The plasmid DNA was detected in the heart, intestine, liver, and gills, but not in muscle tissues. A PCR product of 993 bp, corresponding to the *V. anguillarum* VAOMP gene, was successfully amplified. In contrast, no amplification was observed in fish fed with pellets containing Cs/Tpp-CaP–pcDNA3.1 or Cs/Tpp-CaP–PBS, confirming the specificity of the detection.

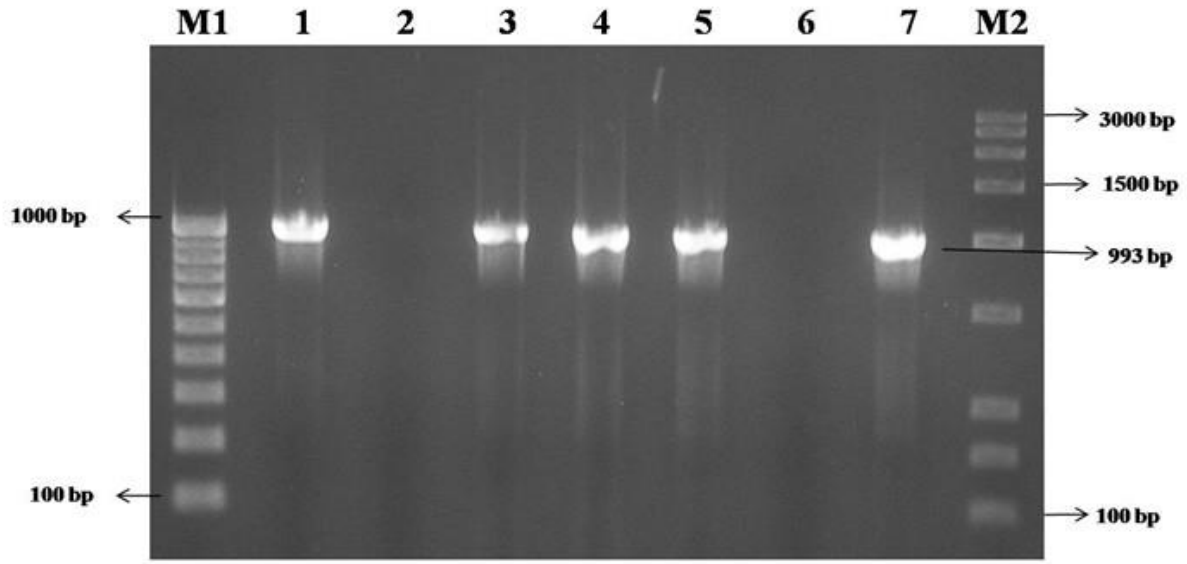



**Figure 4: Agarose gel showing PCR analyses of VAOMP gene of *V. anguillarum* in different organs of fish fed with Cs/Tpp-Cap -pVAOMP DNA. Lane M1 – 100 bp Marker; Lane 1 positive control ( pVAOMP DNA vaccine); Lane 2 – negative control; Lane 3 – heart, Lane 4 – intestine; Lane 5 – gills; Lane 6 - muscle; Lane 7 – Liver; Lane M2 – 3000 bp Marker.**

*Immune gene expression by quantitative real time PCR*

After 10$^{th}$ day of post vaccination, the level of cytokine gene expression such as anti-inflammatory (IL-10) and pro-inflammatory (IL-1β) were significantly changed in the heart, liver, intestine, muscle, and gills of the *C. auratus*. The expression levels of IL10 were significantly upregulated in the heart and muscle of the Group III, whereas in liver and kidney its expression level was significantly downregulated in the Group III. Group II exhibited a significant upregulation of IL-10 expression in the liver, intestine and gills when compared to the control group (Fig 5A). The IL-1β expression levels were significantly upregulated in the heart, liver, intestine, muscle, and gills in the Group III compared to the control. Besides, the Group II showed an upregulation of IL-1β expression in the heart, liver, intestine, and muscle compared

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to the control. On the other hand, the IL-1β expression level was significantly downregulated in gills of the Group II (Fig 5B).

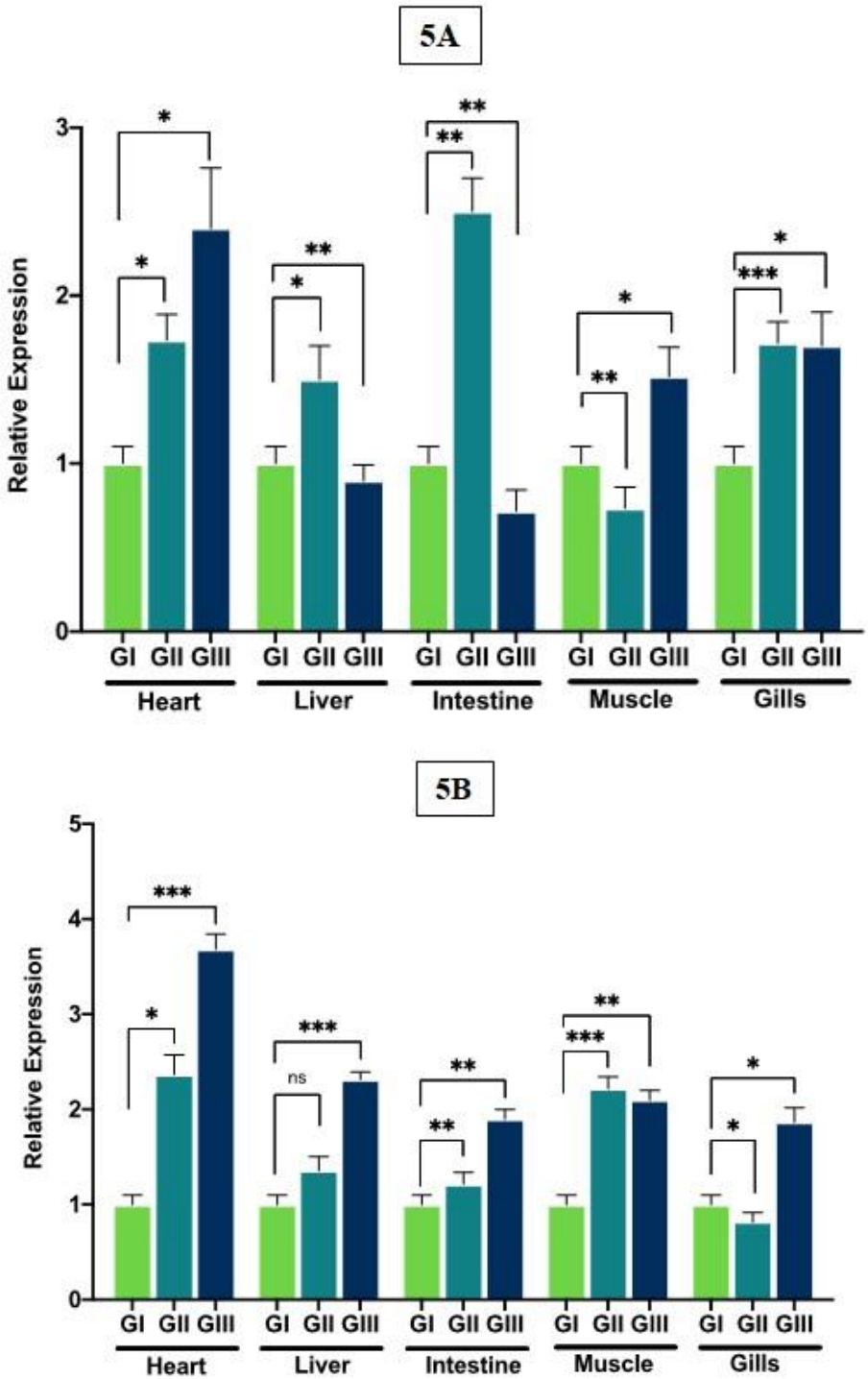



**Figure 5: Expression level of cytokine gene in different organ of *C. auratus* fed with normal feed (GI), pcDNA3.1 (GII) and pVAOMP DNA vaccine nanoformulated with Cs/Tpp-Cap (GIII) on 10th day. The qRT-PCR analysis was performed in triplicates, and each bar were represented as the mean ± SD. (A) Bar graphs showing IL-10 expression in heart, liver, intestine, muscle, and gills. (B) Bar graphs represents IL-1β expression in heart, liver, intestine, muscle, and gills. *$p$<0.05, **$p$<0.01, ***$p$<0.001, indicates a significant difference between the control in response to different groups.**

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*In vivo expression of VAOMP gene by ELISA*

ELISA was performed to assess the in vivo expression of the VAOMP gene in different tissues of fish fed with pVAOMP DNA–Cs/Tpp-CaP. As shown in Figure 6, VAOMP expression was detected in the heart, liver, intestine, and gills, whereas no expression was observed in muscle tissues.

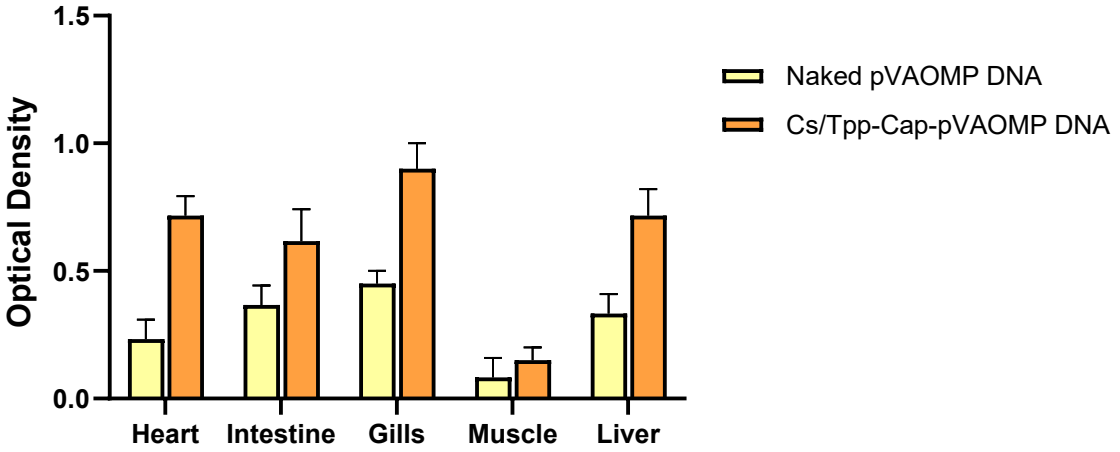



**Figure 6: *In vivo* gene expression of VAOMP protein of *V. anguillarum* in different tissues of the fish fed with Cs/Tpp-Cap -pVAOMP DNA, as determined by ELISA.**

## Discussion

In the present study, oral delivery of the pVAOMP DNA vaccine was successfully achieved using Cs/Tpp-CaP hybrid nanoparticles. The fish fed with pVAOMP DNA encapsulated in Cs/Tpp-CaP nanoparticles expressed the VAOMP gene of *V. anguillarum* in the heart, intestine, liver, and gills, but not in the muscle tissues or in control groups. The Cs/Tpp-CaP nanoparticles were synthesized using low molecular weight chitosan and calcium phosphate through the ionic gelation method and were found to be within the size range of 50–80 nm. These findings are in line with earlier studies, such as Wang *et al.* (2013) who reported the synthesis of Ca–P/CMC hybrid nanoparticles with sizes of approximately 50 nm using low molecular weight chitosan. Similarly, previous research has confirmed the effectiveness of ionic gelation in preparing calcium-based nanoparticles and chitosan–tripolyphosphate complexes (Katas and Alpar 2006; Gan and Wang 2007; Csaba *et al.,* 2009). The versatility of hybrid chitosan nanoparticles has been demonstrated in a range of biomedical applications, including delivery of siRNA for anticancer therapy using PEGylated carboxymethyl chitosan/calcium phosphate (Xie *et al.,* 2014) as well as successful encapsulation and delivery of proteins (Xu and Du 2003), plasmid DNA and oligonucleotides (Csaba *et al.,* 2009), SiRNA (Katas and Alpar 2006; ROJANARATA *et al.,* 2008), and ShRNA. In agreement with these reports, the current study confirms that Cs/Tpp-CaP nanoparticles serve as an efficient oral delivery vehicle for the pVAOMP DNA vaccine in fish. Notably, the findings are consistent with those of Vimal *et al.* (2014), who demonstrated successful delivery of

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plasmid DNA vaccines in multiple fish tissues. Together, these results emphasize the potential of Cs/Tpp-CaP hybrid nanoparticles as a safe and biocompatible platform for mucosal DNA vaccine delivery in aquaculture.

FTIR spectroscopy confirmed the structural organization of the Cs/TPP-CaP hybrid nanoparticles. A strong absorption band at 1642 $cm^{-1}$ was attributed to the $–NH_2$ stretching vibration of chitosan, representing its primary amine groups. After crosslinking with TPP, this band showed a marked reduction in intensity, reflecting the ionic interaction between positively charged amino groups of CS and negatively charged phosphate groups of TPP. Such changes are consistent with earlier reports (Mohammadpourdounighi *et al.,* 2010; Lam *et al.,* 2006; Vimal *et al.,* 2012a) confirming the formation of a polyelectrolyte complex. In addition, the N–H bending vibration shifted from 1595 $cm^{-1}$ in pure CS to 1540 $cm^{-1}$ in the CS/TPP system, further indicating reduced flexibility of amino groups due to crosslinking. The successful incorporation of calcium phosphate was evident from characteristic phosphate ($PO_4^{3-}$) peaks at 582 and 601 $cm^{-1}$, and the carbonate ($CO_3^{2-}$) peak at 872 $cm^{-1}$, typical of substituted apatite structures (Ramachandran *et al.,* 2009). These spectral features confirm that CaP was well integrated within the CS/TPP network, thereby strengthening nanoparticle stability and contributing to its immunostimulatory potential

Morphological characterization confirmed that the Cs/Tpp-CaP nanoparticles synthesized in this study exhibited a predominantly spherical shape with a uniform structure, consistent with earlier findings (Wang *et al.,* 2013; Zhang *et al.,* 2009). The particle size distribution ranged between 50 and 80 nm, as determined by SEM and TEM analysis. Comparable results were reported by Wang *et al.* (2013), who prepared hybrid nanoparticles of calcium phosphate and carboxymethyl chitosan with an average size of approximately 50 nm. In contrast, Zhang *et al*. (2009) developed O-carboxymethyl chitosan/organosilica hybrid nanoparticles with larger diameters of around 200 nm, which were evaluated as non-viral vectors. The smaller size range achieved in the present study suggests that Cs/Tpp-CaP nanoparticles provide favorable characteristics for cellular uptake and effective DNA vaccine delivery in fish.

In this study, the complex coacervation method (Bozkir and Saka 2004) was employed to formulate the pVAOMP DNA–Cs/Tpp-CaP complex. This approach resulted in a high encapsulation efficiency of 85%, demonstrating the strong binding affinity between the DNA and the hybrid nanocarrier. The findings suggest that the combination of calcium phosphate nanoparticles with chitosan tripolyphosphate cross-linking provides a stable and efficient system for DNA encapsulation. Similar strategies have been reported for hybrid nanocarriers, including calcium phosphate/carboxymethyl chitosan for drug delivery (Wang *et al.,* 2013) and PEGylated carboxymethyl chitosan/calcium phosphate for siRNA delivery (Xie *et al.,* 2014). These comparisons reinforce the suitability of Cs/Tpp-CaP nanoparticles as an effective

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oral DNA vaccine delivery platform in aquaculture.

The pVAOMP DNA–Cs/Tpp-CaP complex was prepared using the complex coacervation method (Bozkir and Saka 2004), which achieved a high encapsulation efficiency of 85%. This demonstrates the strong binding affinity and structural compatibility between plasmid DNA and the Cs/Tpp-CaP nanocarrier system. The cross-linking of chitosan tripolyphosphate with calcium phosphate has been shown to provide an effective matrix for nucleic acid delivery, consistent with previous reports on calcium phosphate/carboxymethyl chitosan hybrids for drug delivery (Wang *et al.,* 2013) and PEGylated carboxymethyl chitosan/calcium phosphate nanoparticles for siRNA delivery (Xie *et al.,* 2014). These findings underscore the suitability of Cs/Tpp-CaP nanoparticles as a robust platform for DNA vaccine encapsulation and delivery.

PCR analysis confirmed the successful distribution of the orally administered pVAOMP DNA–Cs/Tpp-CaP vaccine across multiple tissues, including the intestine, heart, gills, and liver, whereas no amplification was observed in muscle tissue. The detection of the vaccine in intestinal tissue is consistent with the oral route of administration, indicating that the nanoparticles were effectively taken up through the gastrointestinal tract. The absence of amplification in muscle tissue may be attributed to lower vascularization or reduced transfection efficiency in this tissue type, which is consistent with previous reports where muscle was found to be a less favorable site for oral or mucosal DNA vaccine uptake (Thirumalaikumar *et al.,* 2021; Vimal *et al.,* 2014). These findings reinforce the tissue-targeting potential of the Cs/Tpp-CaP delivery system and highlight its effectiveness in promoting both local (intestinal) and systemic distribution of the DNA vaccine, while also emphasizing the tissue-specific differences in uptake.

RT-PCR analysis was performed to evaluate the expression of immune-related genes (IL-10 and IL-1β) at 10 days post-vaccination (dpv). The results revealed significantly elevated transcriptional levels of both cytokines, indicating robust immune stimulation in fish administered with the Cs/Tpp-CaP–pVAOMP DNA vaccine. Notably, gene expression was strongly detected in the intestine, heart, gills, liver, and muscle, suggesting that the nanocarrier facilitated systemic delivery and effective immune activation. These findings are in agreement with earlier studies employing chitosan-based nanoparticles and microspheres, which also demonstrated enhanced cytokine expression and immune stimulation (Kanellos *et al.,* 1999; Tian *et al.,* 2008; Vimal *et al.,* 2014; Thirumalaikumar *et al.,* 2021).

The expression of VAOMP protein in different tissues was further validated by ELISA, confirming that the oral delivery of pVAOMP DNA encapsulated in Cs/Tpp-CaP nanoparticles resulted in in vivo expression of the encoded antigen (Tian *et al.,* 2008; Vimal *et al.,* 2012b). The successful expression of exogenous genes highlights the efficiency of Cs/Tpp-CaP nanoparticles as a DNA delivery platform. This is consistent with the mechanism proposed by Leong *et al.* (1998), where

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chitosan-based nanoparticles transport nucleic acids across epithelial barriers via both paracellular and transcellular pathways. Taken together, the present findings clearly demonstrate that Cs/Tpp-CaP nanoparticles function as a safe, biocompatible, and biodegradable non-viral delivery system. Their ability to encapsulate and protect negatively charged molecules such as DNA, proteins, and double-stranded RNA, while ensuring efficient uptake and gene expression in multiple tissues, underscores their potential application in aquaculture vaccine development.

## Conclusions

The present study demonstrated that Cs/Tpp-CaP nanoparticles can be successfully formulated through electrostatic interactions between positively charged chitosan–TPP–CaP and negatively charged DNA molecules, leading to the self-assembly of stable hybrid nanocarriers. Gene distribution and expression analyses confirmed the effective delivery of the pVAOMP DNA vaccine, with detectable expression of the VAOMP gene in multiple tissues and significant upregulation of immune-related genes. These results highlight the potential of Cs/Tpp-CaP nanoparticles as an innovative and biocompatible platform for oral DNA vaccine delivery. Given their stability, high encapsulation efficiency, and ability to promote both gene transfer and immune activation, Cs/Tpp-CaP hybrid nanoparticles represent a promising strategy for the development of oral DNA and other non-viral gene vaccines in aquaculture.

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## Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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