Immuno Protective Efficacy of a DNA Vaccine Encoding the *Edwardsiella piscicida* Flagellin Encapsulated Chitosan Nanoparticles

***Chamilani Nikapitiya, Dananjaya, S.H.S, and Mahanama De Zoysa**

College of Veterinary Medicine and Research Institute of Veterinary Medicine,
Chungnam National University, Daejeon 34134, Republic of Korea.
E-mail: Chamilani14@gmail.com

Abstract

In this study, we constructed *E. piscicida* flagellin DNA encapsulated chitosan nanoparticles based DNA vaccine, named pDNA-flagellin-CNPs. The encapsulated pDNA-flagellin-CNPs were nano sized and had high stability and encapsulation efficiency. *In vitro* transfection results demonstrated that pDNA- flagellin-CNPs expressed in human cells (HEK293T) and maintained its bioactivity. *In vitro* cell cytotoxicity analysis confirmed the safety of using pDNA-flagellin-CNPs. When zebrafish were treated with pDNA-flagellin-CNPs, the genes involve in pattern recognition (toll like receptors; *tlr5a*, *tlr5b*, and *myd88*), inflammatory (*tnfα*, *il1β*,, *il8,* and *il12*), and transcription factor (*nf-κb*,) responses were significantly upregulated (day 1 and 3 post treatment; dpt) compared to rest of the groups (negative control, naked pDNA, and pDNA-CNPs). Moreover, when zebrafish challenged with *E. piscicida* at 7 dpt, higher cumulative survival (>50%) was observed in the pDNA-flagellin-CNPs compared to the pDNA-CNPs and the naive group. In conclusion, the constructed pDNA-flagellin-CNPs are safe to use and produce efficient innate immune responses responsible for activating humoral responses. It further suggests that CNPs are promising carriers for plasmid DNA encapsulation and efficient DNA vaccine delivery for vaccinate the fish against *E. piscicida*.

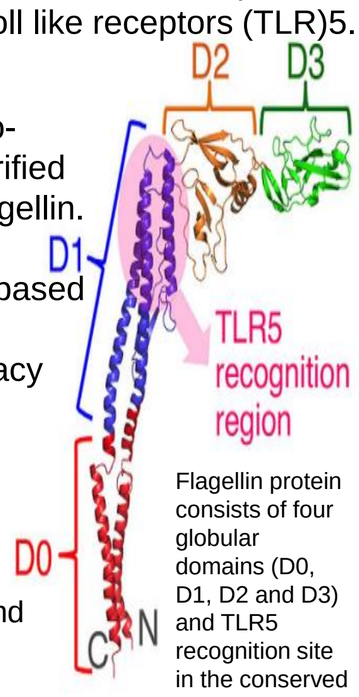
Background

- Pathogenic *Edwardsiella piscicida* (Gram negative bacteria) has a broad host range and causes Edwardsiellosis.
- DNA vaccination is one of the promising alternatives over traditional vaccines for controlling many pathogenic infections.
- When DNA plasmids delivered as a naked plasmids, it could undergo degradation by nucleases, thus cause inefficient delivery to immune cells.
- Hence, for efficient DNA vaccine delivery to fish, novel delivery strategies are required.
- Chitosan nanoparticles (CNPs) as nano-scaled carriers showed excellent value due to its biodegradable and biocompatibility and efficient gene delivery capacity.

- Flagellin is a main component of bacterial flagella and stimulates host defense responses in many organisms via toll like receptors (TLR)5.
- Researchers have investigated the immuno-protective efficacy of purified *E. tarda* recombinant flagellin.
- However, DNA vaccine based pDNA-flagellin-CNPs immuno-protection efficacy has not been studied.

➢ Objectives:

- Investigating the physico-chemical, biocompatible and immunoprotective efficacy of constructed pDNA-flagellin-CNPs.

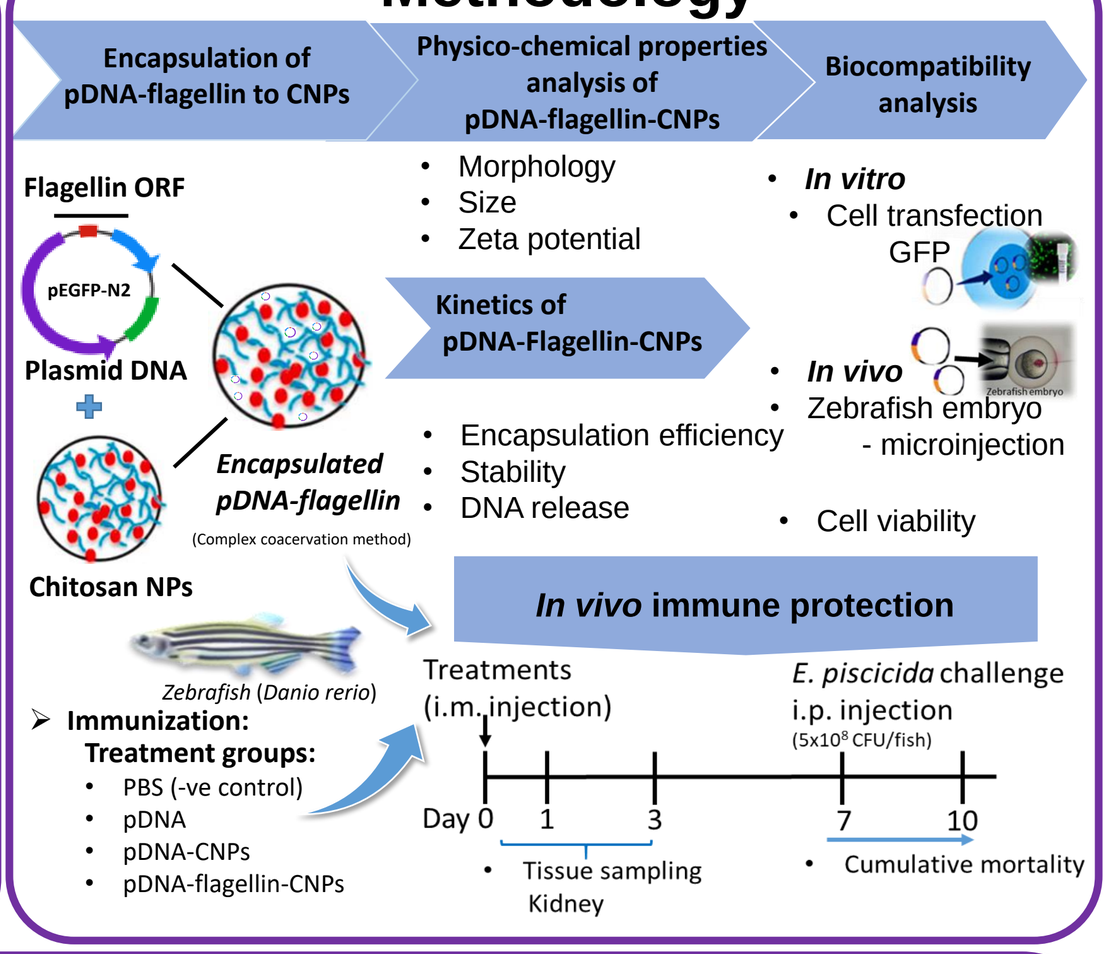


Flagellin protein consists of four globular domains (D0, D1, D2 and D3) and TLR5 recognition site in the conserved D1 domain.

Flagellin structure

Lu & Swartz, 2016, Sci. rep., 6, 18379

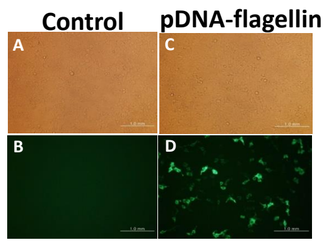
Methodology



Results

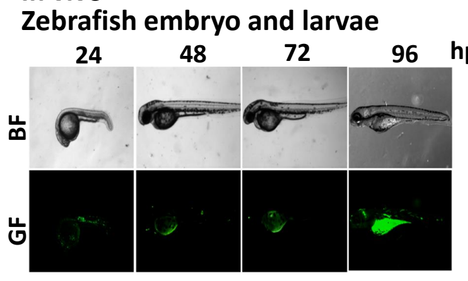
➢ *In-vitro* and *in vivo* expression of pDNA-flagellin

In vitro
HEK293T cells



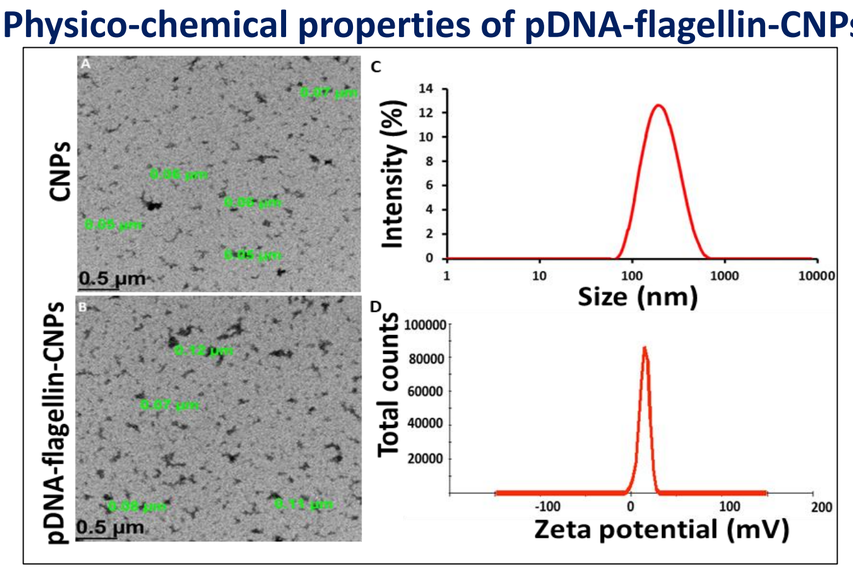
In-vitro expression of control and pDNA-flagellin in HEK293 cells. EGFP expression in HEK293 cells at 48 h post transfection when plasmid was delivered by Polyethylenimine. (A) Control, no plasmid transfected bright field images and (B) fluorescence detection, (C) pDNA transfected bright field images and (D) fluorescence detection.

In vivo
Zebrafish embryo and larvae



pDNA-flagellin (20 pg) was injected per embryo using microinjection and GFP was observed with the development stages.

➢ Physico-chemical properties of pDNA-flagellin-CNPs



- Transmission Electron Microscopy micrographs of (A) CNPs; (B) pDNA-flagellin-CNPs. (C) zeta potential and (D) particle size distribution of pDNA- flagellin-CNPs (N/P = 3).

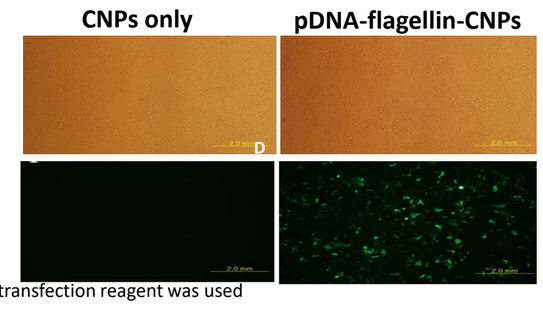
➢ Evaluation of loading efficiency

N/P	Loading efficiency (%)	Particle size (nm)	Zeta potential (mv)
0.25	60.34±3.62	188.25±3.89	10.25±1.29
0.5	72.45±2.34	182.21±2.34	10.93±2.24
1	80.46±2.86	175.26±3.45	12.15±1.72
2	84.42±4.5	170.26±4.15	12.95±1.15
3	93.24±3.24	165.26±3.45	14.25±2.85
4	91.36±1.25	161.32±2.18	16.12±0.95

- Values are presented as mean ± standard deviation of five experiments in each group.

- Highest loading efficiency (93.24 ± 3.24%) observed at N/P = 3.
- Particle size: 165.26 ± 3.45 nm and zeta potential : 14.25 ± 2.85 mV.

➢ *In vitro* Expression of pDNA-Flagellin-CNPs

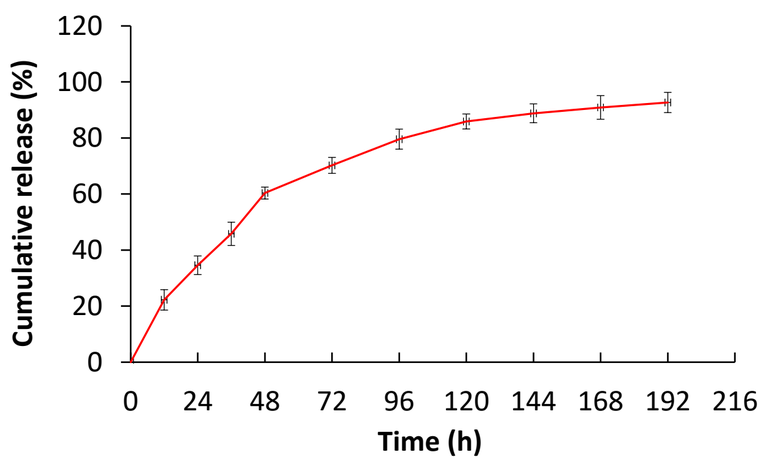


No transfection reagent was used

In-vitro expression of CNPs and pDNA-flagellin-CNPs in HEK293 cells at 48 h post transfection. A) CNPs, no plasmid transfected bright field images, and (B) fluorescence detection. (C) pDNA-flagellin-CNPs transfected bright field images, and (D) fluorescence detection. The scale bars are 2.0 mm

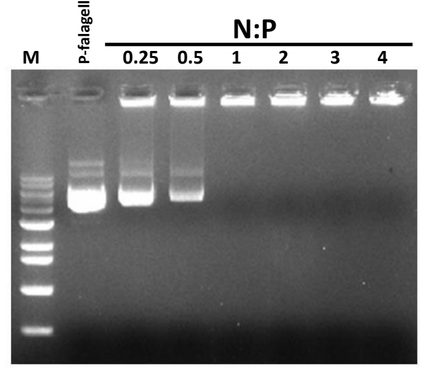
- Cells have uptake the pDNA-Flagellin-CNPs, due to the effect of the CNPs encapsulation that act as a DNA delivery carrier.

➢ pDNA-flagellin loading and release of CNPs

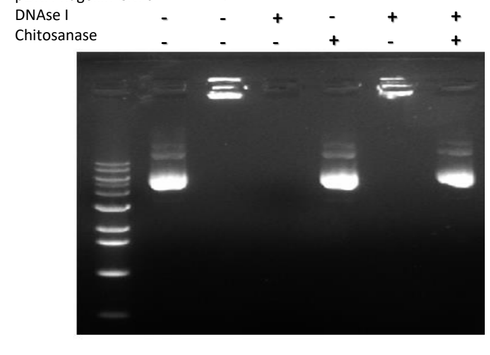


- *In vitro* release profiles of the pDNA-flagellin from pDNA-flagellin-CNPs; Values are expressed as means ± SD (n=3).

➢ Gel retarding analysis

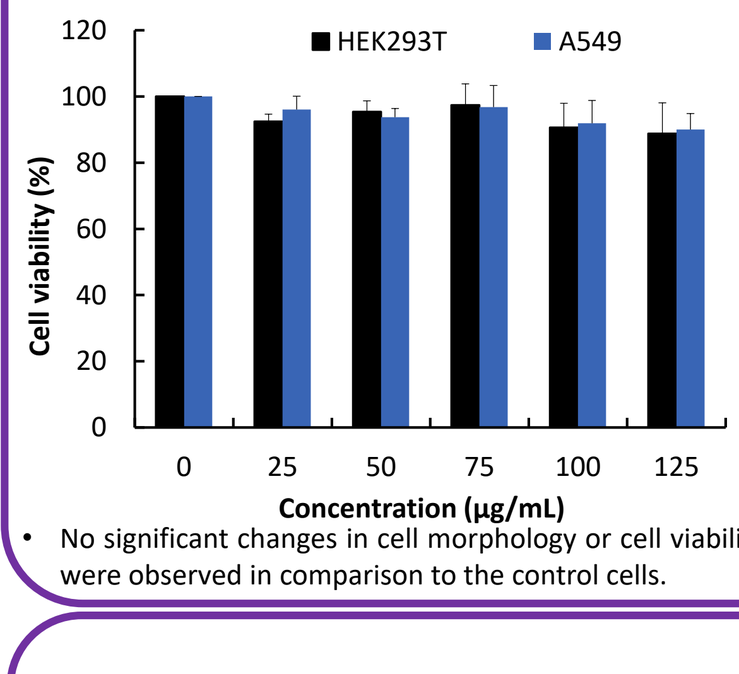


➢ Electrophoretic mobility analysis



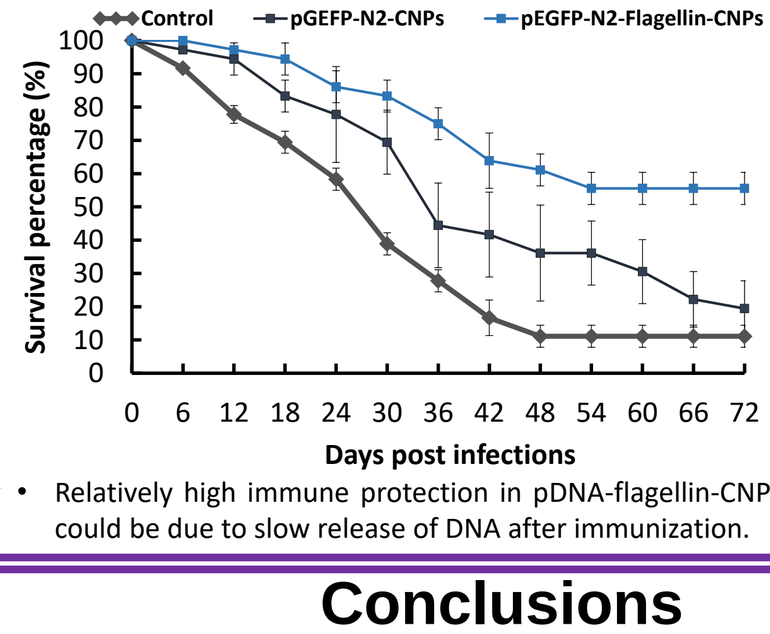
(A) Gel retarding analysis of pDNA-flagellin-CNPs. Lane 1; 1 kb marker, Lane 2; naked pDNA-flagellin, Lanes 3-8; NPs prepared at N/P ratios of 0.25, 0.5, 1, 2, 3 and 4, respectively. (B) Electrophoretic mobility analysis of pDNA-flagellin-CNPs to determine DNA integrity. Lane 1; 1kb marker, Lane 2; naked pDNA- flagellin; Lane 3; pDNA-flagellin-CNPs, Lane 4; naked pDNA- flagellin + DNAse I, Lane 5; pDNA-flagellin-CNPs + chitosanase, Lane 6; pDNA- flagellin-CNPs + DNAse I, Lane 7; pDNA-flagellin-CNPs + DNAse I + chitosanase.

➢ Cytotoxicity of pDNA-flagellin-CNPs



- No significant changes in cell morphology or cell viability were observed in comparison to the control cells.

➢ Protection of Zebrafish from *E. piscicida* challenge after vaccination of fish



- Relatively high immune protection in pDNA-flagellin-CNPs could be due to slow release of DNA after immunization.

➢ Effect of pDNA-flagellin-CNPs immunization on zebrafish immune gene expression

Gene category	Gene	Day	Kidney			Muscle		
			pDNA	pDNA-CNPs	pDNA-flagellin-CNPs	pDNA	pDNA-CNPs	pDNA-flagellin-CNPs
Toll like receptors	*tlr5a*	1	1.23	1.81	2.34	1.38	2.16	2.85
		3	1.71	1.92	2.94	0.99	2.25	3.88
	tlr5b	1	1.22	2.02	3.01	1.09	1.38	2.82
		3	1.59	2.77	4.82	0.88	1.51	2.89
Toll like receptor adaptor protein	*myd88*	1	1.94	2.7	4.31	0.98	1.22	4.86
		3	1.49	5.69	15.35	2.54	13.31	18.54
Pro and anti-inflammatory cytokines & signaling molecules	*tnf-α*	1	1.71	2.81	3.94	0.77	1.72	2.19
		3	2.83	3.45	5.68	0.77	1.72	2.19
	Il-1β	1	1.62	1.78	2.62	1.16	1.57	2.33
		3	1.38	2.64	9.42	1.67	3.1	3.82
	nf-κb	1	1.72	1.52	3.06	1.38	2.36	2.85
		3	0.98	2.02	4.14	0.99	2.45	3.18
	Il-6	1	1.33	1.54	2.63	0.77	1.72	2.19
		3	2.33	8.46	25.42	1.28	1.85	2.61
	Il-8	1	1.94	2.7	4.31	1.62	1.83	2.60
		3	1.86	8.68	14.32	1.47	2.24	2.96
	Il-12	1	1.22	1.34	2.2	1.16	1.57	2.33
		3	1.24	1.54	3.25	1.16	1.77	2.91

Relative expression -fold
≥ 2.5- fold
≥ 2 - < 2.49 - fold
≥ 0.5 - < 2-fold

Relative Expression folds were calculated based on the PBS treated group (–ve control; 1-fold)

- TLR5 and MyD88 genes were upregulated in kidney and muscle of zebrafish after immunization. This suggests that enhanced innate immune responses, could initiate adaptive responses efficiently.

Conclusions

- The constructed plasmid contains flagellin gene of *E. piscicida* and it was successfully encapsulated with CNPs.
- Encapsulated pDNA-flagellin-CNPs had a mean diameter of 165.26, a zeta potential of 14.25 mV, and morphology with no aggregations. The encapsulation was confirmed to protect the DNA plasmid from DNase I digestion and presence of green fluorescence indicated that the pDNA-Flagellin-CNPs express in HEK293T cells and maintain good bioactivity.
- Importantly, Intramuscularly immunization with pDNA-flagellin-CNPs could partially protect zebrafish from highly virulent *E. piscicida* infection (55% of RPS), which was significant with survival analysis.
- Compared to the zebrafish immunized with the encapsulated control plasmid, *in vivo* immunization showed that fish immunized with the pDNA-flagellin-CNPs had better immune responses and this may be due to release of the plasmid DNA was prolonged.
- This study evident that flagellin DNA plasmid encapsulated with CNPs are potent immunization candidates against *E. piscicida* infection and provides strategies for the further development of novel vaccines encapsulated in CNPs.

Reference: Lu, Y., & Swartz, J.R. (2016). Functional properties of flagellin as a stimulator of innate immunity (Sci. Rep. 6: 18379).

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