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Delivery of double stranded RNA by *Macrobrachium rosenbergii* nodavirus-like particles to protect shrimp from white spot syndrome virus

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ABSTRACT

We tested the use of *Macrobrachium rosenbergii* nodavirus-like particles (MrNv-VLPs) as a delivery mechanism to carry therapeutic agents against white spot syndrome in shrimp. We used constructed double-stranded RNA called VP28 (VP28 dsRNA) against WSSV envelope genes to confer protection against the pathogen. Results showed that MrNv-VLP was able to encapsulate VP28 dsRNA. Using enhanced green fluorescent protein (EGFP) as a reporter, we found that VLP penetrated various shrimp tissues including the muscle, hepatopancreas, and gill. A statistically significant relative survival rate of 44.5% was obtained in the group of shrimp receiving encapsulated VP28 dsRNA-VLP after WSSV challenge as compared to 100% mortality in the control shrimp at 7 days post-infection. Shrimp treated with EGFP dsRNA loaded into MrNv-VLPs showed relatively similar motility rates as those of controls. Moreover, MrNv-VLP encapsulation improved VP28 dsRNA efficiency against WSSV. A higher survival rate of 16.7% was observed in the group of shrimp receiving encapsulated VP28 dsRNA-VLP when compared to those receiving naked VP28 dsRNA. These results indicate that MrNv-VLP is a good candidate for use as a therapeutic delivery system against shrimp diseases due to its self-reassembly property, broad target of various shrimp tissues and immune enhancement.

1. Introduction

In recent years, nanotechnology of delivery systems has attracted increased interest in many areas of science, agriculture, and allied fields, including aquaculture and fisheries. Ideally, delivery systems should be able to target the relevant antigen-production region, allow interaction with the cells of interest and induce minimal immune response. Several reports have demonstrated the feasibility of shrimp vaccination and gene delivery using nanocontainers derived from non-viral synthetic biopolymers such as poly(lactic-co-glycolic) acid (PLGA) (Nikolaev et al., 1997) and chitosan (Ravi et al., 2009), which are able to deliver genes to specific tissues of marine animals with minimal toxicity to the host cell. Chitosan is safer than PLGA as no organic solvent is required during antigen entrapment. Because it is positively charged, chitosan has the ability to bind with negatively charged immunogenic DNA/RNA (Saroja et al., 2011). It can also open up tight junctions and increase the paracellular transport of antigens (van der Lubben et al., 2001). Alternatively, protein-based biological containers have been developed as active compound delivery systems and are marketed as a medical innovation. Each rational design is a nanotechnology-based development that aims to target cellular scale, hence called nanotherapy or nanovaccine. Many of which are specified for treating or killing the target cells or organs (Uchida et al., 2007; Wörsdörfer et al., 2011). The natural protein cage candidates chosen for this purpose are mostly viral and bacteriophage capsids, which form virus-like particles (VLPs) and modified for attaching or attacking the target cells (Stephanopoulos et al., 2010; Uchida et al., 2007). VLPs are biocompatible and naturally functional in the similar ways as those infecting virions which are able to recognize host cells through receptor mediated interaction, fuse with cell membrane entry (Ludwig and Wagner, 2007). In addition, based on their self-assembly and disassembly properties, VLPs have the ability to encapsulate and deliver plasmid DNA into cells *in vitro* and *in vivo* as demonstrated for both mammalian infecting

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viruses and shrimp nodaviruses (Inoue et al., 2008; Jariyapong et al., 2014).

The structural and physicochemical properties of nodaviruses, causative agents of infectious diseases in aquaculture, have been studied extensively to gain fundamental knowledge of RNA viruses and development of antiviral immunity (Hou et al., 2009; Johnson et al., 2001; Lin et al., 2001). Their recombinant capsid protein can be produced using either baculovirus or *Escherichia coli* (Sahul Hameed and Bonami, 2012; Shetty et al., 2012; Tang et al., 2001) and self-assembly to form VLPs. Many reports have demonstrated the utilization of VLPs derived from flock house nodavirus (FHV) as an antigen-presenting system for many therapeutic materials with variable degrees of success (Buratti et al., 1997; Chen et al., 2006; Manayani et al., 2007). Capsid protein of *Macrobrachium rosenbergii* nodavirus (MrNv) has been expressed recently and self-assembled into VLPs with a diameter of approximately 30 ± 3 nm (Goh et al., 2011; Jariyapong et al., 2014). Due to the high presence of basic amino acids at the *N*-terminal end, MrNv-VLPs possess a positive charge (Goh et al., 2014), which effectively binds to the negative charge of nucleic acid, causing them to fold inward to the capsid quaternary structure.

Our previous study reported the encapsulation and delivery of plasmid DNA carrying GFP by MrNv-VLP *in vitro* (Jariyapong et al., 2014). Based on the above properties, MrNv-VLP has been considered a promising nanocontainer to encapsulate therapeutic agents such as double-stranded RNA (dsRNA) or short oligonucleotides against bacterial and viral diseases. Previous studies have successfully demonstrated the use of RNA-based vaccines to protect shrimp from WSSV infection (Kim et al., 2007; Xu et al., 2007). An antiviral effect of dsRNA or silencing RNA (siRNA) synthesized from the VP28 gene of WSSV has been reported and administered to shrimp before WSSV challenge (Sarathi et al., 2008; Westenberg et al., 2005). In this study, we explore the possibility of MrNv-VLP as a nanocarrier to deliver VP28 dsRNA to prevent viral infection in shrimp.

2. Methods

2.1. Expression and purification of MrNv-VLP

Protein expression and purification were carried out according to previously described methods (Jariyapong et al., 2014). Briefly, the pET16b-MrNv constructs were transformed into *E. coli* BL21 (DE3) and incubated at 37 °C overnight, followed by further incubation at 25 °C until absorbance reached 0.6–0.8 at 600 nm (A_{600}). Recombinant *E. coli* cells, in which pET16b-MrNv tagged with six consecutive histidines had been induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG), were harvested by centrifugation at 5400 ×*g* for 10 min. The pellet was re-suspended in phosphate buffered saline (PBS) containing 500 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated at 100 Hz for 10 cycles. The sonicated samples were pelleted at 12,000 ×*g* for 10 min and the supernatant was collected and loaded onto a HisTrap™ FF column pre-packed with nickel sepharose resin (GE Healthcare, Waukesha, WI, USA). After washes with 20 and 50 mM imidazole in 500 mM NaCl, the bound proteins were eluted with elution buffer (250 mM imidazole, 500 mM NaCl, pH 7.4) and further subjected to discontinuous sucrose gradient centrifugation. The fractionated MrNv capsid proteins were collected for protein concentration assay using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at −20 °C until used.

2.2. Production of VP28 dsRNA

To produce VP28 dsRNA, the total cDNA from the hemocyte of WSSV infected shrimp were amplified by the specific primers for the VP28 gene (Table 1). The PCR amplification protocol comprised preheating at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 min, 72 °C for 30 s, and a final extension of 72 °C for 5 min. The PCR products were further amplified using the same thermal profile and two sets of specific primer incorporating T7 promoter with gene specific primer for VP28, as shown in Table 1. The dsDNA products were checked on 1% agarose gels and purified by column purification using the Amicon® Ultra-0.5 (30K membrane) device (Merck, Darmstadt, Germany) according to the manufacturer's instructions. The purified dsDNA were subjected to *in vitro* transcription using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The dsRNA were purified using the phenol–chloroform–isoamyl protocol. Concentration of dsRNA was measured by a spectrophotometer and stored at −80 °C until use.

Table 1
Primer pairs used in this study.

Gene	Primer sequence
VP28	(F) 5′-ATGGATCTTTCTTTCACTCTTTC-3′
	(R) 5′-TTACTCGGTCTCAGTGCCAG-3′
T7VP28F	(F) 5′-TAATACGACTCACTATAGGGAT GGATCTTTCTTTCACTCTTTC-3′
	(R) 5′-TTACTCGGTCTCAGTGCCAG-3′
T7VP28R	(F) 5′-ATGGATCTTTCTTTCACTCTTTC-3′
	(R) 5′-TAATACGACTCACTATAGGG TTACTCGGTCTCAGTGCCAG-3′
EGFP	(F) 5′-CATGGTCCTGCTGGAGTTCGTG-3′
	(R) 5′-CGTCGCCGTCCAGCTCGACCAG-3′
WSSV	(F) 5′-CCGACGCCAAGGGAACTGT-3′
	(R) 5′-TTCAGATTCGTTACCGTTTCCA-3′
WSSV probe	CTTCAGCCATGCCAGCCGTCT TCCA

2.3. Encapsulation of VP28 dsRNA

Disassembly of MrNv-VLP was performed using the protocol employed in the researchers' previous study (Jariyapong et al., 2014). In brief, purified MrNv-VLPs were incubated with a disassembly buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM ethyleneglycoltetraacetic acid (EGTA), and 20 mM dithiothreitol (DTT) for 1 h at room temperature. For encapsulation, an equal concentration of VP28 dsRNA was added to the disassembled VLPs in the disassembly buffer. To resume VLP assembly, concentration of $CaCl_2$ was slowly increased by adding it to the mixture to reach a final concentration of 5 mM and was followed by further incubation for 1 h. The mixture was separated by ultracentrifugation at 200,000 ×*g* for 2 h. The pellet of encapsulated VP28 dsRNA-VLP was collected and used for calculating encapsulation efficiency and delivery assay *in vivo* as described below.

2.4. Calculation of VP28 dsRNA loaded into MrNv VLP

The encapsulated VP28 dsRNA-VLPs were heated in the presence of SDS-PAGE loading dye at 95 °C for 5 min to denature the assembled VLPs. The released VP28 dsRNA and the serially diluted purified VP28 dsRNA (3–15 μg) were electrophoresed in 1% agarose gel, stained with ethidium bromide, and visualized by UV light. The band intensities of the loaded VP28 dsRNA and known amounts of the respective VP28 dsRNA were quantified densitometrically using an ImageScanner (Amersham Pharmaica Biotech, Piscataway, NJ, USA). The amount of loaded VP28 dsRNA after encapsulation was then calculated using an equation generated from already known amounts of VP28 dsRNA. The encapsulation efficiency (EE) was calculated as follows:

$$EE = \frac{\text{Load amount of VP28 dsRNA in pellet}}{\text{Total amount of VP28 dsRNA}} \times 100.$$